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# STUDIES ON THE INTERACTION BETWEEN PYRIDINE-HEMIN AND HYDROGEN PEROXIDE OR OXYGEN. I. SPECTROSCOPIC OBSERVATIONS ON THE PROCESS OF VERDOHEMOCHROME FORMATION WITH SPECIAL REFERENCE TO ITS QUANTITATIVE TREATMENT

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(Received for publication, April 14, 1951)

The specific catalytic activities of hemin derivatives on hydrogen peroxide or oxygen have long been discussed in the enzyme chemistry both physiologically and chemically. It is well known, on the other hand, that hemin derivatives can easily be degraded into some green pigments by the action of hydrogen peroxide or oxygen in the presence of certain reductants (*1–7*). It might, therefore, be assumed that the catalytic actions of hemin derivatives would be accompanied by some destruction of their own molecules. For the study of the reactions between hemin derivatives and hydrogen peroxide or oxygen, it will be important to point out such a characteristic interaction between the reactants.

The present study deals with more intimate study on process of verdohemochrome formation from pyridine-hemin complex by the action of oxygen and ascorbic acid, which was once presented by Lemberg, *et al.* (*5*).

## EXPERIMENTAL METHODS

*Material*—$1.1 \times 10^{-4}$ *M* pyridine-hemin solution. Recrystallized hemin, prepared from ox blood by the method of Willstätter (*8*), was dissolved in 20% pyridine.

Crystalline pure L-ascorbic acid was dissolved in redistilled water to a desired concentration at each use.

*Procedure*—Throughout the experiments in this paper the changes of absorption spectrum were traced by the use of wave-length spectro-

meter combined with "Spekker" spectrophotometer along with the reactions which took place directly in the absorption cell. 3.0 ml. of pyridine-hemin was pipetted into the absorption cell (1 cm.), covered with liquid paraffin to avoid any contact with air. Oxygen in the solution was driven out by bubbling with $N_2$ gas through a glass capillary for about 1.5 minutes, and then 0.3 ml. of ascorbid acid solution was added. Immediately afterwards, the cell was placed in the light way of spectrophotometer and then aerated through an inserted glass capillary by the use of Mariotte-bottle. The capillary was fine and the bottle was placed sufficiently high so as to keep the aeration velocity nearly constant throughout the reaction, *i.e.*, 100 ml. of air per 10 minutes, in an average. Aeration was stopped during every reading.

## RESULTS

*Changes of the Absorption Curve Throughout the Reaction Process.*—Along with the reaction, the absorption curve of the reaction mixture changes as shown in Fig. 1, in which 0 indicates the absorption curve of $1 \times 10^{-4}$ *M* pyridine-hemochrome (dipyridino-ferroporphyrin) before aeration.

On aeration for 2 minutes, there appeared a new absorption in the range of 600–640 m$\mu$ (Fig. 1, 2). In the green region, however, its three maxima of 557, 525 and 480 m$\mu$, were markedly decreased. On 6-minutes aeration (Fig. 1, 6) more increasing changes were observed and the absorption maximum in the red was still persisting distinctly at 630 m$\mu$. On 10-minutes aeration (Fig. 1, 10), however, maximum in the red shifted to 645 m$\mu$ and the maximum at 557 m$\mu$ became scarcely recognizable. Moreover, in the green region, a new maximum at 530 m$\mu$ appeared in place of 525 m$\mu$ maximum and another new maximum at 500 m$\mu$.

By continued aeration, the maximum in the red, increasing in its optical density, moved gradually toward a longer wave-length side and, at the same time, the maximal absorption of 530 m$\mu$ increased further. After a 30-minutes aeration (Fig. 1, 30), the maximum in the red was fixed finally at 656 m$\mu$, indicating the final stage of the reaction process. This last product is the verdohemochrome of Lemberg, which was first described by H. Fischer as "grünes Haemin."

Transition of absorption maxima during the aeration are summarized in Table I (time in minutes).

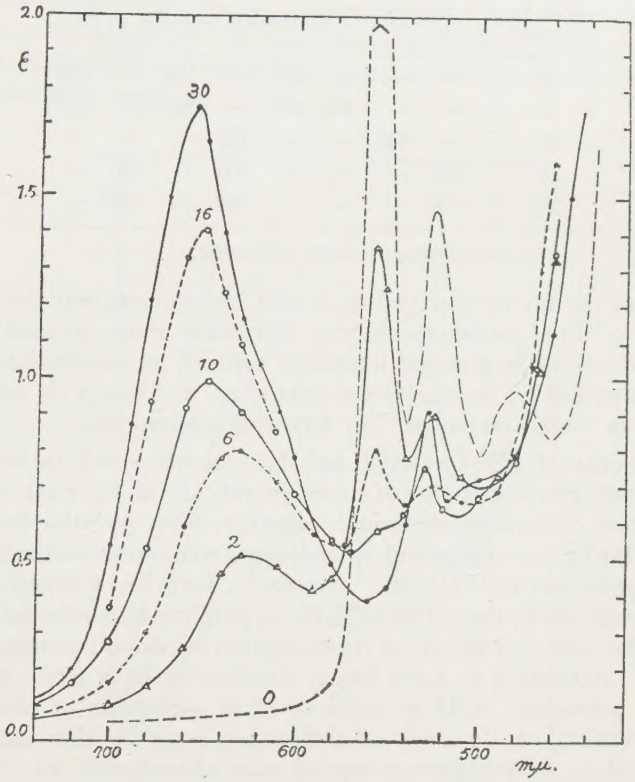


FIG. 1. Changes of the absorption curve throughout the reaction process.

3.0 ml. of $1.1 \times 10^{-4}$ *M* hemin in 20% aequeous pyridine.
0.3 ml. of $5.5 \times 10^{-4}$ *M* ascorbic acid in water.
T = 10°, Aeration: 100 ml. per 10 minutes.
Numbers in the Figure represent the minutes of aeration.

TABLE I

*Transition of Absorption Maxima During the Aeration*

| Time in minutes | Positions of absorption maxima (mμ) |
|---|---|
| 0 | — — — 557 — 525 — 480 |
| 2 | — — 630 (557) — (525) — (480) |
| 10 | — 645 — — (530) — — — |
| 16 | 656 — — — 530 — 500 — |
| 30 | 656 — — — 530 — 500 — |

( )—diminishing or newly appearing.

From the above observation, it will be assumed that the reaction process of the verdohemochrome formation may proceed in two stages; the reaction proceeds seemingly through an intermediate, which is characterized by its absorption maximum at 630 mμ (it will be expressed as "630-compound" in following descriptions).

*Properties of 630-Compound and Verdohemochrome*—Lemberg had already noticed the existence of a considerably stable intermediate among the process of verdohemochrome formation from pyridine-hemin and Libowitzky also recognized an analogous compound similarly derived from coprohemin methyl ester. Moreover, Lemberg found the compound available by the action of $H_2O_2$ on pyridine-hemin in the presence of ascorbic acid and described its absorption maximum as noticeable at 639 mμ. According to Lemberg, it seems to be a ferric oxyhemochrome possessing -OH in place of H of α-methene of protohemin. Libowitzky, on the basis of elementary analysis, also assumed his compound to be iso-hydroxy-copro-1-ester chlorohemin in which one hydrogen atom in one of the methene bridges has been substituted by a hydroxyl group.

Following Lemberg's method, the authors obtained a compound characterized by its absorption maximum at 630 mμ from pyridine-hemin by adding a certain amount of hydrogen peroxide in the presence of ascorbic acid (the compound corresponds to that of Lemberg, for which a maximum at 639 mμ was described by him).

The absorption curve of the nearly pure 630-compound obtained above are given in Fig. 2, I. There exists no recognizable absorption maximum in the green region any more. 630-compound can hardly be reduced by ascorbic acid, but is easily reduced by $Na_2S_2O_4$ and, as

the result of reduction, the absorption in the red disappears corresponding to the recovery of two absorption maxima at 557 and 525 mμ in the green (Fig. 2, II). On account of the densities of these two absorptions by 630-compound, however, $\varepsilon_{557}$ is smaller then $\varepsilon_{525}$, contrary to that of pyridine-hemochrome. In accordance with the absorption change, the color of the solution turns from green of 630-compound to orange red of its reduced stage.

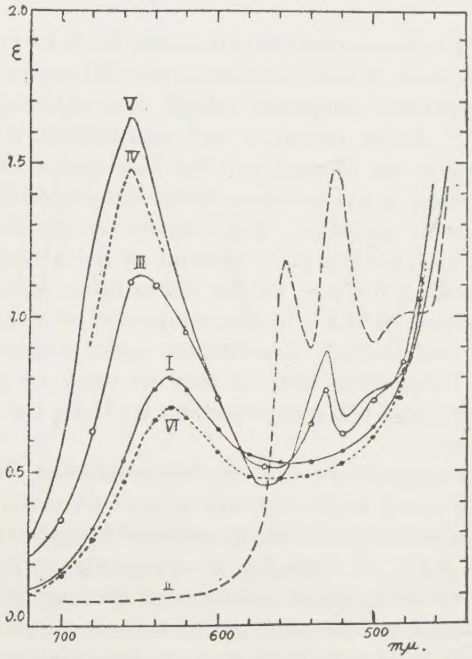


FIG. 2. Absorption curves of 630-compound and its derivatives.

I: 630-Compound ($Fe^{+++}$) formed by the reaction of $1\times10^{-4}$ $M$ pyridine-hemin, $1.5\times10^{-2}$ $M$ ascorbic acid and $5\times10^{-3}$ $M$ $H_2O_2$.
T=8.5°.

II: reduced 630-compound ($Fe^{++}$).

III: on 5-minute aeration of I.

IV: on 12-minute aeration of I.

V: after 30-minute aeration of I.

VI: after addition of 0.2 ml. of $\frac{1.5}{20}$ $M$ $H_2O_2$ to I.

The absorption of 630 mμ maximum in Fig. 2, I increases gradually toward 656 mμ by aeration until it reaches the typical absorption curve of verdohemochrome (Fig. 2, III–V). It is important to point out that, in the experiment described in Fig. 1, the absorption maximum in the red still persisted at 630 mμ after 6 minutes' aeration with its density of $\varepsilon_{630}=0.85$, but in the case of Fig. 2, in which the reaction took place by direct addition of $H_2O_2$, absorption maximum has already moved to 650 mμ on aeration of only 5 minutes, giving its density of $\varepsilon_{650}=1.13$.

In spite of his reasonable interpretation, Lemberg left untouched the final identification of both substances, the 639 mμ compound and the proposed intermediate compound which was obtained by the direct action of $H_2O_2$. Above results of our experiments, however, may be sufficient to suggest the identity of these two compounds and that the reaction mechanism of the pyridine-hemin decomposition in both cases to be identical in its 1st stage. The authors are not in agreement with the description of Lemberg on account of the absorption maximum at 639 mμ, instead of 630 mμ, for the intermediate substance indicated.

By the addition of $H_2O_2$ to 630-compound, verdohemochrome will not be formed and instead, it undergoes some unusual decomposition (Fig. 2, VI). From these facts, it may be assumed that verdohemochrome is formed from 630-compound not by $H_2O_2$ but by the action of molecular oxygen.

It must be pointed out that verdohemochrome, the final product of the system, is much more registant against reduction than 630-compound. Both substances will not be reduced by ascorbic acid but both will be by $Na_2S_2O_4$, if $Na_2S_2O_4$ is sufficiently active. If, however, $Na_2S_2O_4$ is less effective, which will be often the case by a long preservation, 630-compound will be easily reduced by it, but verdohemochrome will not. It may be of some interest to say here that the redoxpotentials of hemin derivatives can vary according to the change of constitution in the porphyrine ring.

As the result of reduction, the characteristic absorption maximum of verdohemochrome at 656 mμ separates itself immediately into two maxima of 650 mμ and 683 mμ and later, these two absorptions decrease gradually. Absorption at 683 mμ decreases more promptly and disappears after several minutes (Fig. 3, III). At the same time, the color of the reaction mixture turns from green to yellow green. In the region of shorter wave-length, Soret band shifts considerably to the longer wave-length side, and the maxima of 500 mμ and 530 mμ

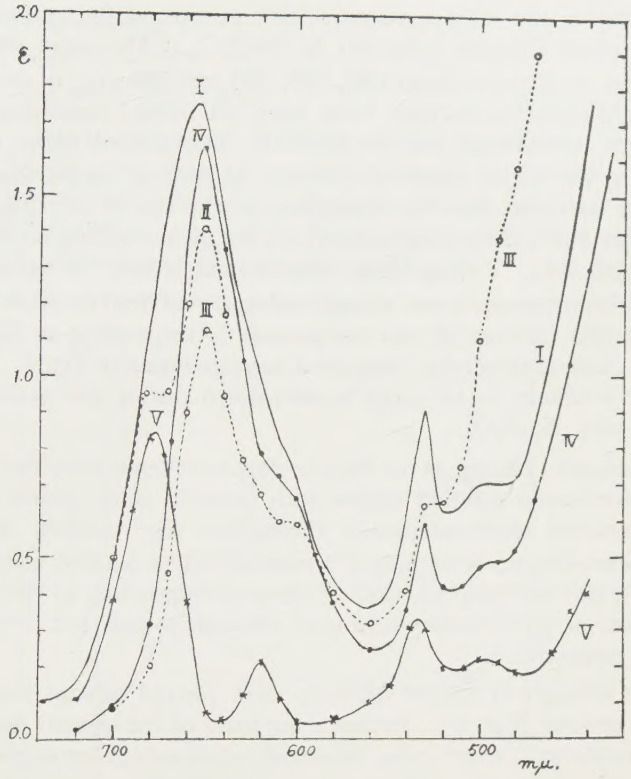


FIG. 3. Effect of $Na_2S_2O_4$ upon verdohemochrome.

I: verdohemochrome ($1 \times 10^{-4}$ *M*).
II: directly after the addition of $Na_2S_2O_4$ to I.
III: after 10 minutes' keeping of II without contact of air.
IV: after 15 minutes' aeration of III.
V: differences of optical densities in each wave-lengths between I and IV.

become scarcely recognizable. If the solution is aerated again, the color of blue green will reappear for a moment and the whole absorption curve approaches that of the original verdohemochrome, if not completely (Fig. 3, IV). Differences of the optical densities between these two absorptions of the original verdohemochrome and that of the recovered substance by aeration in its final state are calculated and plotted in each wave-lengths as shown by Fig. 3, V. This calculated curve

corresponds to the disappearance of that portion of the optical density brought about through reduction by $Na_2S_2O_4$. The latter shows four absorption maxima at about 680, 620, 535 and 500 m$\mu$; in comparison with verdohemochrome they have been all moved somewhat toward the longer wave-length side in general. The plotted curve is distinguished by the higher grade of similarity to that of verdohemochrome. It should be noted that the absorption in the red of the original verdohemochrome is always unsymmetric; it has some swelling on the longer wave-length side. Taking these observations, it may be assumed that the verdohemochrome is not a single substance of final reaction product, but a certain mixture of two compounds corresponding to Fig. 3, IV and V. Among these, the compound corresponding to Fig. 3, V would have the tendency to be easily broken down during the process of reduction with $Na_2S_2O_4$.

*Quantitative Tracing of the Reaction Process*—Along with the aeration of pyridine-hemin solution added with ascorbic acid, optical densities were measured spectroscopically throughout the reaction, in which certain wave-lengths were properly marked. The marked wave-lengths were 557, 630 and 656 m$\mu$, each of them corresponding to the maximal absorption of pyridine-hemochrome, 630-compound and verdohemochrome, respectively.

The changes of optical densities were plotted against the time of reaction process (Fig. 4). Between the ratio of the optical densities in each wave-length, there exist following relationships corresponding to the named substances. These were established experimentally empirical,

$\varepsilon_{630}\,m\mu / \varepsilon_{656}\,m\mu = 0.67$ in verdohemochrome and

$\varepsilon_{656}\,m\mu / \varepsilon_{630}\,m\mu = 0.69$ in 630-compound. (This was determined by several samples of 630-compound, prepared from pyridine-hemin by the addition of $H_2O_2$ in the presence of ascorbic acid.)

As to the pyridine-hemochrome, the absorptions of both at 630m$\mu$ and 656 m$\mu$ are so feeble as to be negligible in our experiments. At the concentration of $1\times10^{-4}\,M$, $\varepsilon_{557}=2.8$ in pyridine-hemochrome, nearly 0.52 in 630-compound and still a little smaller in verdohemochrome. Presumably, pyridine-hemin is kept in $Fe^{++}$ state throughout the reaction and the additive effect of absorption of each substances should be secured, the following equations is available to demonstrate the actual absorption of each substances at a certain time.

$$\varepsilon_{630} = x + 0.67\,y$$
$$\varepsilon_{656} = y + 0.69\,x$$
$$\varepsilon_{557} = z + \frac{0.52}{2.8}(2.8 - z)$$

Where x: actual absorption of 630-compound at 630 m$\mu$.
y: actual absorption of verdohemochrome at 656 m$\mu$.
z: actual absorption of pyridine-hemochrome at 557 m$\mu$.

By applying these equations to Fig. 4, actual changes of concentration of each substances were calculated (Fig. 5).

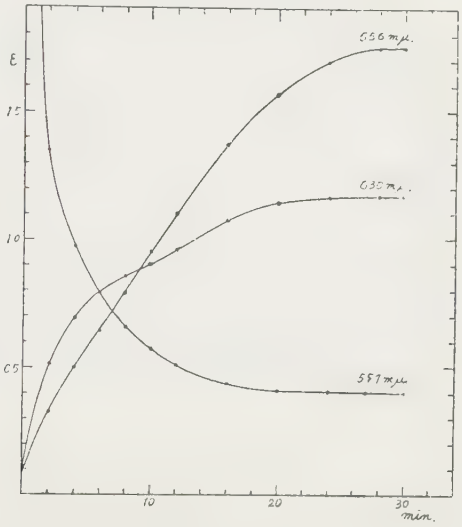


Fig. 4. Changes of optical densities in each marked wavelength.
Experimental conditions, same as in Fig. 1.

The reaction proceeds in this system obviously in two stages: Pyridine-hemin → 630-compound → verdohemochrome.

The extinction coefficient of each compound is thus empirically available, and the curves in Fig. 5 will be now expressed in terms of absolute concentration (Fig. 6). While verdohemochrome should be formed through 630-compound, the sum of the concentrations of both substances (dotted line in Fig. 6) should correspond to the value of 630-compound formed at each time. If this dotted curve will be compared with the degradation curve of pyridine-hemochrome, it will be noticed that the latter runs somewhat below the theoretical value. This

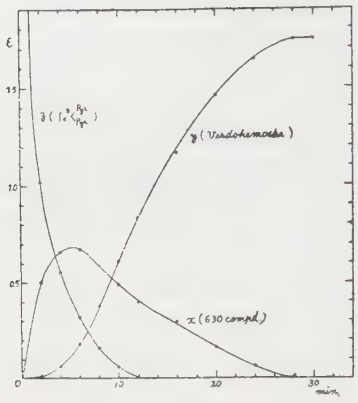


FIG. 5. Changes of the actual absorption by each substance. calculation from FIG. 4.

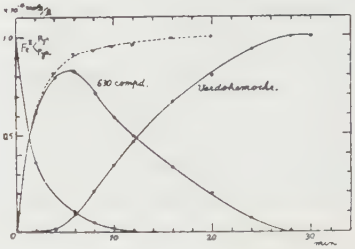


FIG. 6. Changes of the absolute concentration, calculated from FIG. 5.

Dotted line represents the sum of the concentrations of 630-compound and verdohemochrome.

difference will be attributed to the fact that some pyridine-hemichrome (dipyridino-ferriporphyrin) are more or less formed in this reaction. The formation, if minor, of pyridine-hemichrome during the reaction process can be assured by the slight recovery of $\varepsilon_{557}$ or $\varepsilon_{525}$, which can be noticed every time by stopping the aeration.

## DISCUSSION

The decomposition of pyridine-hemin in this reaction system proceeds obviously in two stages; first, the formation of 630-compound, and the second, of verdohemochrome.

630-compound can be obtained from pyridine-hemin both by the aeration and addition of $H_2O_2$, each in the presence of ascorbic acid. For the analysis of the reaction process, it seems necessary to point out some important observations as follows:

(a) It was often noticed by longer keeping in open atmosphere that the color of pyridine-hemichrome solution changes to some tone of green, but the resulting absorption curve differs entirely from that of 630-compound.

(b) The changes of color toward green will be also observable if $H_2O_2$ is added to pyridine-hemichrome without any reductant but, as its absorption curve thus obtained will be different from that of 630-

compound, it should be assumed that the reaction process in this case obviously does not proceed in a similar course to that of 630-compound.

(c) No gas bubbles will be noticed when $H_2O_2$ is added to pyridine-hemin in the presence of ascorbic acid. Therefore the molecular oxygen should by no means be taken into consideration as a possibility of interposing, in the reaction process.

On the basis of above observations, it can be assumed that 630-compound will not be produced by the action merely of $O_2$ or $H_2O_2$ unless in the presence of certain reducing agents. As a conclusion, the mode of 630-compound formation in this reaction system may be regarded as follows: First, $H_2O_2$ is produced from ascorbic acid and $O_2$ by the catalytic action of pyridine-hemin, and second, $H_2O_2$ thus produced, acting on pyridine-hemin (still doubtful whether at $Fe^{++}$ or $Fe^{+++}$), forms 630-compound. The assumption had already been argued early by Lemberg, but he was not able directly to verify the specific rôle of $H_2O_2$ in the course of this reaction. Our observations, as pointed by Fig. 2, however, would be sufficient to admit such an interpretation.

The molecular oxygen seems to be necessary especially for the process of verdohemochrome formation from 630-compound. The reaction proceeds throughout the whole process significantly simply and completely quantitatively as is clearly demonstrated by Fig. 6. These facts may offer some important background for the analysis of the reaction mechanism.

Taking above experimental facts into consideration, it may be now assumed that the decomposition of pyridine-hemin occurs obviously as a result of its catalytic function. In other words, hemin derivative will be decomposed oxidatively by the action of $H_2O_2$ or $O_2$ which is catalytically activated by hemin derivatives themselves. Especially, the 630-compound would be formed through some mechanism which seems to be very similar to the peroxidatic reaction. Thus an intimate connection would be brought forth between the oxidative decomposition of the porphyrin ring of hemin and the fact that hemin contains iron. For example, according to Haurowitz (*9*), coordination complex compound of protoporphyrin with Cu, Co or Mg, displaying no specific reaction with dilute $H_2O_2$, will not be affected by a dilute $H_2O_2$. In addition, according to Cook (*10*), iron-phthalocyanine possesses a remarkable catalase action, and in the course of its process, iron-phthalocyanine itself will be decomposed to a certain colorless substance. Above phenomena can be attributed to the catalytic function which is displayed by a special state of iron.

SUMMARY

The reaction process of verdohemochrome formation from pyridine-hemin was spectroscopically studied, which takes place through the aeration in the presence of ascorbic acid. The results are summarized as follows:

1. The reaction in this system proceeds in two stages: pyridine-hemin→630-compound (an intermediate substance giving its maximal absorption at 630 mμ)→verdohemochrome.

2. 630-compound is formed by the action of $H_2O_2$ under the presence of ascorbic acid.

3. The process of 630-compound → verdohemochrome requires particularly the presence of molecular oxygen.

4. Method of the quantitative analysis of the reaction process was established.

5. The reaction proceeds completely quantitatively throughout the whole process.

ACKNOWLEDGEMENT

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# ON THE KINETICS OF THE HUMAN BLOOD CHOLINESTERASE

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The human blood cholinesterase can be classified into an erythrocytes specific cholinesterase and a serum non-specific cholinesterase, and one of the differences of these two types have been shown clearly on the pS-activity curve (Fig. 1) as described first by Alles and Hawes (*1*) and later, by Tamai (*2*). Namely, the optimum substrate concentration of erythrocyte cholinesterase is considerably lower than that of serum cholinesterase and, in addition, the former shows a significant inhibition by the excess of substrate.

It is still disputed on what mechanism such a difference is based. Bisseger and Zeller (*3*) assumed that, in the case of a specific cholinesterase, enzyme-substrate complex will be formed in two sites of the substrate molecule, one at nitrogen atom and another at acetyl group, while in the case of a non-specific cholinesterase, the complex compound will be formed only at the acetyl group, thus the latter would be capable of splitting non-cholinesters as well.

Koelle and Gilman (*4*) also assumed that the specific cholinesterase will combine with acetylcholine molecule at two sites. In a lower substrate concentration, the enzymic reaction proceeds through ES complex initially produced. In a higher concentration, however, an inactive complex ESS may be formed exhibiting a substrate inhibition.

For the studies on the essential feature of the differences of two types of enzymes, the relationship between the reaction velocity of the enzymic process and the reaction of enzyme with substrate will be of extreme importance. The present paper offers some clue on this point based on the experiments upon the kinetics of the two types of cholinestrease in human erythrocytes and serum and also upon the inhibition by eserine.

Wright (*5*) analysed the reaction of both cholinesterases applying the equation of Lineweaver and Burk (*6*) and explained the differences of inhibitory action by antimalarial drugs. As to the action

of eserine on canine serum cholinesterase, Eadie (*7*) and Goldstein (*8*) are not in agreement regarding the number of eserine molecule with which the enzyme would combine. Bain (*9*) recently dealt with the mechanism of the inhibition of rat brain cholinesterase by eserine and alkylphosphates, but attaining no conclusion at all.

## METHOD

*Enzyme Preparation*—About 5 ml. of blood was drawn from antecubital vein, defibrinated and exactly 2 ml. of it was centrifuged. The serum was then diluted two-fold with a Ringer solution of the following prescription.

| | | |
|---|---|---|
| 100.0 ml. | 0.90 g./dl. | NaCl |
| 2.0 ml. | 1.15 g./dl. | KCl |
| 2.0 ml. | 1.22 g./dl. | $CaCl_2$ |
| 20.0 ml. | 1.30 g./dl. | $NaHCO_3$ |

Erythrocyte was washed three times with the Ringer solution and finally made up exactly to 10 ml. of suspension in a Ringer solution. 0.1 ml. of them were used for the experiments.

*Substrate Solution*—Acetylcholine " Takeda " was used as a substrate in Ringer solution. Taking consideration of autohydrolysis, it is first dissolved in a Ringer solution not containing $NaHCO_3$. Bicarbonate solution is then added at the ratio of five to one, just before the experiments.

*Inhibitor*—Eserine sulfate " Merck " was used as the inhibitor.

*Procedure*—The enzyme activity was measured at 37.5° by the manometric method introduced by Ammon (*10*). Frequency of shaking; 85 per minute. The acetylcholine-Ringer solution was placed in the main chamber and the enzyme solution in the side-arm of the vessel. Gas mixture, $CO_2$: $N_2$=5: 95.

Rate of hydrolysis was measured by the reading of the manometer by every ten minutes after mixing the contents. The velocity was then expressed by cmm. of $CO_2$ evolved during 10 minutes of the second 10 minutes.

In order to exclude the influence of autohydrolysis of acetylcholine on the value of measurements, the readings were corrected by performing a control.

## EXPERIMENTAL RESULTS AND ITS ANALYSIS

*pS-Activity Curve of Erythrocytes and Serum Cholinesterase*—

(a) Process of reaction:

The substrate concentration was varied from $3 \times 10^{-3} M$ to $5 \times 10^{-4} M$. Each of them was used as an initial concentration. By taking measurements of $CO_2$ evolved during 10 minutes, at each initial concentration, the relationship between log (a –x) and the time was plotted (Figs. 2 and 3). In these figures, the substrate concentration has been expressed, as a matter of convenience, by the calculated quantity of $CO_2$ to be evolved.

In both erythrocytes and serum, the relationship between log (a–x)

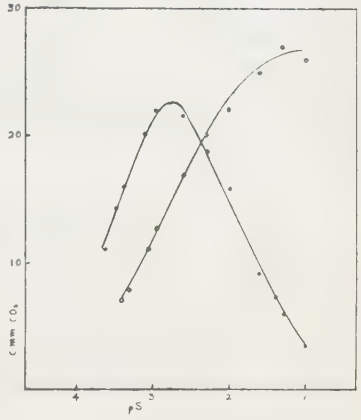


FIG. 1. pS-Activity curve for acetylcholine hydrolysis by human erythrocyte and serum cholinesterase.

●——●: erythrocyte, o——o: serum.

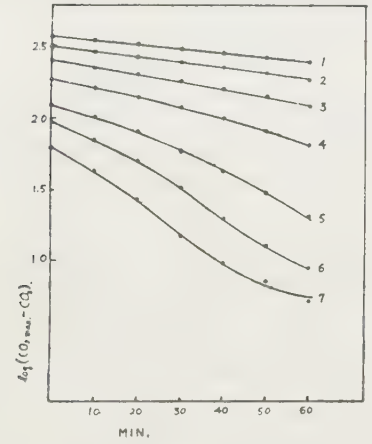


FIG. 2. Change of log (a–x) with time for erythrocyte cholinesterase.

Curve 1: Initial substrate concentration of 0.003 *M*; curve 2: 0.0025 *M*; Curve 3: 0.002 *M*; curve 4: 0.0015 *M*; curve 5: 0.001 *M*; curve 6: 0.00075 *M*; curve 7: 0.0005 *M*.

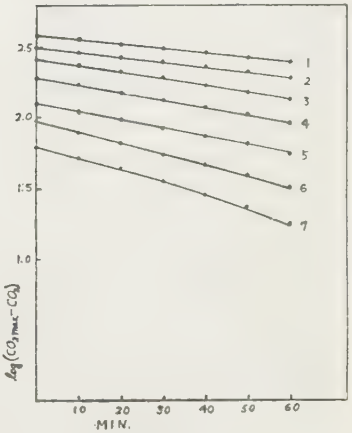


FIG. 3. Change of log (a–x) with time for serum chloinesterase.

Curve numbers: Initial substrate concentration as noted in FIG. 2.

and the time has been thus expressed by the sigmoid curves with a mild slope.

Presuming it as an inhibitory effect of substrate in its higher concentration involving ESS complex, which is inactive as assumed by Koelle and Gilman (*4*), the whole reaction may be represented as follows:

$$E+S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \quad \text{.....(Eq. 1)}$$

$$ES \xrightarrow[k_3]{} E+P \quad \text{.....(Eq. 2)}$$

$$ES+S \underset{K'm}{\rightleftarrows} ESS \quad \text{.....(Eq. 3)}$$

where E, S and P are cholinesterase, acetylcholine and final reaction product, respectively.

The decomposition of acetylcholine by cholinesterase of erythrocytes and serum seems essentially to be the same, and if the substrate concentration is lower than the optiumm substrate concentration, each reaction will proceed mainly according to the Eq. 1 and Eq. 2, and the reaction Eq. 3 will then interpose thereto along with the higher substrate concentration.

Presuming the dissociation constants of the complex of ES and ESS in the erythrocyte cholinesterase to be smaller than those of the serum cholinesterase, the differences of the phenomena of two types of enzymes can be simply explained.

(b) Michaelis-constant of cholinesterase of erythrocytes and serum:

In both cholinesterase of erythrocytes and serum, the reaction proceeds according to Eq. 1 and Eq. 2 in case the substrate concentration is lower than the optimum substrate concentration, so that the reaction velocity v can be expressed as follows:

$$v=k_3 [\Sigma E] [S]/(Km+[S]) \quad \text{.....(Eq. 4)}$$

where $[\Sigma E]$ is the total concentration of cholinesterase and Km the Michaelis constant. The limiting velocity, Vmax can be introduced as follows through Eq. 4:

$$Vmax=k_3 [\Sigma E] \quad \text{.....(Eq. 5)}$$

Consequently, Eq. 6 may be derived from Eq. 4 and Eq. 5:

$$Vmax/v=1+Km/[S] \quad \text{.....(Eq. 6)}$$

According to the actual measurements, both cholinesterase of erythrocytes and serum shows a linear relationship between 1/v and 1/[S] in the case of a lower substrate concentration (cf. Figs. 4 and 5).

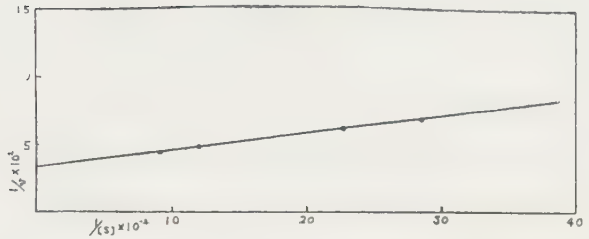


FIG. 4. Evaluation of Km for erythrocyte cholinesterase from Eq. 6. $Km = 3.7 \times 10^{-4}$ mole/lit.

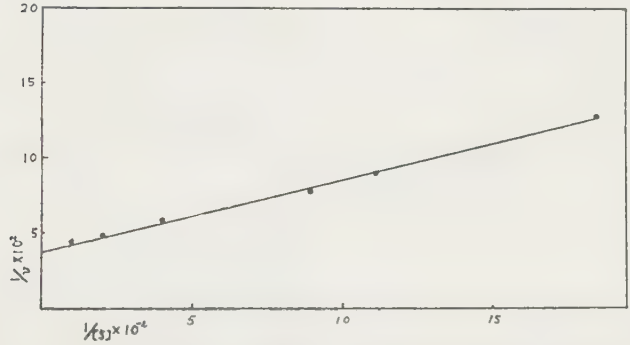


FIG. 5. Evaluation of Km for serum cholinesterase from Eq. 6. $Km = 1.2 \times 10^{-3}$ mole/lit.

The theoretical relationship of Eq. 6 is thus supported by the experimental results.

The values obtained for Km of two types of enzymes through the relationship thereof, are $3.7 \times 10^{-4}$ mole/lit. for erythrocytes and $1.2 \times 10^{-3}$ mole/lit. for serum. Wright (*5*) reported the values as $2.0 \times 10^{-4}$ mole/lit. on the former and $1.6 \times 10^{-3}$ mole/lit. on the latter.

(c) Inhibition by excess of substrate.

With increasing substrate concentration, Eq. 3 interposes thereto. Therefore

$$[\Sigma E] = [E] + [ES] + [ES][S]/K'm$$

where K'm is the dissociation constant of ESS.

As [E] will be negligible in case the substrate concentration becomes higher,

$$v = k_3[\Sigma E]\ K'm/(K'm + [S]) \quad \text{..... (Eq. 7)}$$

If the substrate concentration becomes infinitely small, as K′m/(K′m+[S]) to be a unit, the reaction velocity V′max may be represented as follows:

$$V'max = k_3[\Sigma E]$$

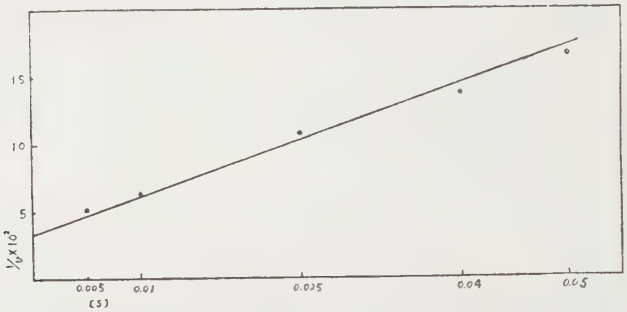


Fig. 6. Evaluation of K′m for erythrocyte cholinesterase from Eq. 8. $Km' = 1.2 \times 10^{-2}$ mole/lit.

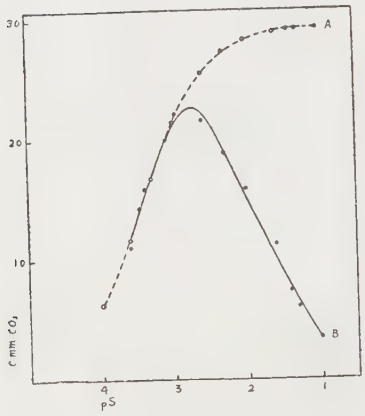


Fig. 7. pS-Activity curve of erythrocyte cholinesterase: experimental curve compared with theoretical.

Curve A: Theoretical, plotted by calculations based on the Km value.

Curve B: Actual measurements.

Consequently,

$$V'max/v = 1 + [S]/K'm \quad \text{.....(Eq. 8)}$$

Through actual measurements in higher substrate concentration, a linear relationship between 1/v and [S] could be established in accordance with the theoretical Eq. 8.

The value obtained thus for K′m is $1.2 \times 10^{-2}$ mole/lit.

If it may be presumed that, even in higher substrate concentration, the reaction might proceed without any inhibition by excess substrate, then the reaction may proceed along with the presumable curve of Fig. 7 A which was drawn from a calculation based upon the Km value found.

Taking $v_0$ as the reaction velocity expressed by Fig. 7 A, and v the actual reaction rate measured as Fig. 7 B, the degree of inhibition H by substrate will be represented as follows:

$$H=1-v/v_0 \quad \text{.....(Eq. 9)}$$

The relationship between H and pS (-log[S]) will be plotted on a sigmoid curve of the first order, as shown by Fig. 8.

Therefore, the following equation will be introduced.

$$H=\frac{[S]}{\phi+[S]} \quad \text{.....(Eq. 10)}$$

From this, the value of $\phi$ could be calculated as $1.3\times10^{-2}$ mole/lit.

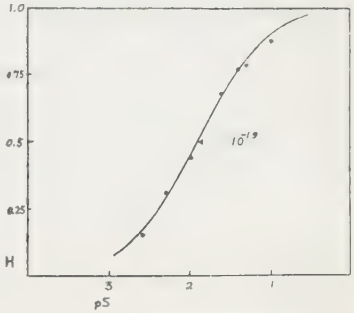


Fig. 8. Inhibition-pS-curve by excess substrate.
$\phi=1.3\times10^{-2}$ mole/lit.

Now, the value of $v_0$ and v in Eq. 9 can be replaced by Eq. 4 and Eq. 7, and the following equation will be introduced.

$$H=\frac{[S]-Km\cdot K'm/[S]}{K'm+[S]} \quad \text{..... (Eq. 11)}$$

If the substrate concentration becomes high enough as $Km\cdot K'm/[S]$ to be negligible, Eq. 11 may be simplified as follows:

$$H=\frac{[S]}{K'm+[S]} \quad \text{..... (Eq. 12)}$$

Thus $\phi$ in Eq. 10 is represented here by K'm. The relation introduced is supported by experimental results in which the values of K'm and $\phi$ were found to be approximately equal to those as described above. The inhibition by excess substrate should be thus attributed again to the formation of ESS compound. It was thus clearly demonstrated that the provisionally offered reaction formulas can be satisfactory supported by the experimental evidence. The difference of these two types of cholinesterase, which is expressed by their pS-activity curve, will be attributed, as a matter of fact, to the difference of the Michaelis constant of each enzyme, seemingly based on the character of their protein fraction. The marked inclination of erythrocyte cholinesterase to substrate inhibition will indicate its easy reactivity to ESS, an inactive complex.

It is, however, still a problem of the future whether the characters of enzymes, which are to be classified into specific and non-specific cholinesterase, should also be attributed to the difference of the same constant or not.

*Mechanism of Inhibition by Eserine*—The enzyme was added to the substrate of certain concentration mixed previously with eserine in vari-

ous concentrations, and the activities were plotted against the time. Results are shown in Figs. 9 and 10. The figures were obtained with the substrate concentration of $2\times10^{-2}M$ with serum, and $5\times10^{-3}M$ with erythrocyte. On the addition of eserine, the reaction does not proceed linearly until 20′ were reached after shaking. The same relationship has been found even by varied substrate concentrations.

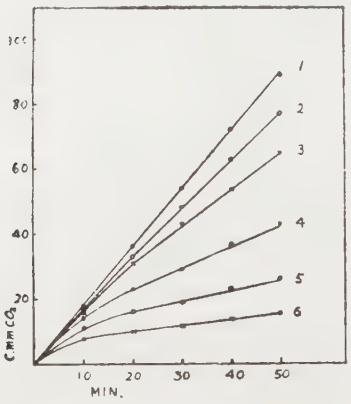


Fig. 9. The activity-time curves of erythrocyte cholinesterase.

Substrate concentration, 0.005 *M*. Eserine in varied concentrations as follows. Curve 1: without eserine; curve 2: $5\times10^{-8}M$; curve 3: $10^{-7}M$; curve 4: $2.5\times10^{-7}M$; curve 5: $5\times10^{-7}M$; curve 6: $10^{-6}M$.

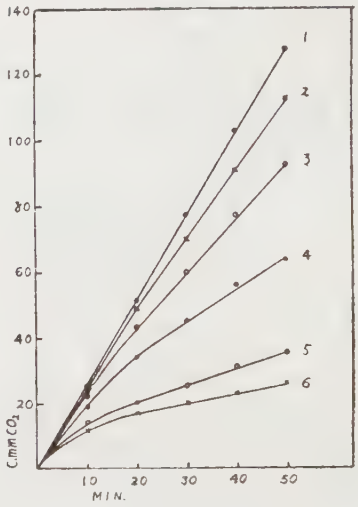


Fig. 10. The activity-time curves of serum cholinesterase.

Substantrate concentration, 0.02 *M*. Curve number: concentration of eserine as noted in Fig. 9.

The linear parts of the figure were taken for analysis, in which the reaction equilibrium seems to be settled. The reaction velocity of the system added with eserine will be expressed by vg and that without eserine, by $v_0$. They should be indicated by a tangent on the angle between each straight line and abscissa. The inhibition by eserine, expressed by H, may be represented as follows:

$$H=1-vg/v_0 \quad \text{..... (Eq. 13)}$$

With varying concentrations of eserine [G] under certain substrate

concentrations, the relationship between H and pG(-log [G]) could be plotted as shown in Figs. 11 and 12.

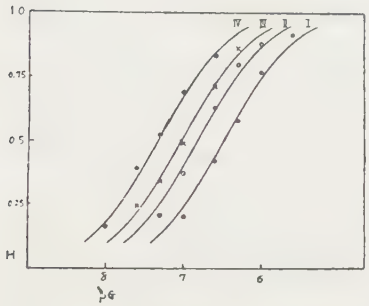


FIG. 11. Inhibition-pG-curve of erythrocyte cholinesterase by eserine.

Curve I: Substrate concentration of 0.01 *M;* curve II: 0.005 *M;* curve III: 0.0025 *M;* curve IV: 0.001 *M.*

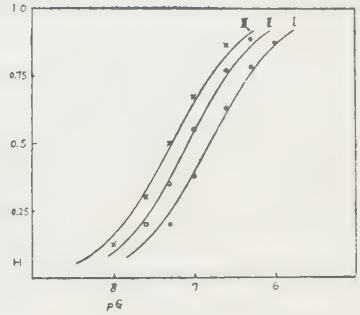


FIG. 12. Inhibition-pG-curve of serum cholinesterase by eserine.

Curve I: Substrate concentration of 0.02 *M;* curve II: 0.01 *M;* curve III: 0.005 *M.*

As to the relationship between H and pG, a sigmoid curve of the first order was obtained by both the cholinesterase of erythrocytes and serum. Under each substrate concentration, the indicated relationship can be represented as follows:

$$H=\frac{[G]}{\phi+[G]} \quad \text{(Eq. 14)}$$

Herewith, the values of $\phi$ under each substrate concentration were found to be as in Table I.

TABLE I

*Experimental Data of $\phi$ Values under Varied Substrate Concentrations*

| Molarity of substrate | 0.02 | 0.01 | 0.005 | 0.0025 | 0.001 |
|---|---|---|---|---|---|
| Erythrocyte | —— | $3.3\times10^{-7}$ | $1.54\times10^{-7}$ | $0.92\times10^{-7}$ | $0.46\times10^{-7}$ |
| Serum | $1.58\times10^{-7}$ | $0.82\times10^{-7}$ | $0.54\times10^{-7}$ | —— | —— |

As the Eq. 14 has been proved by the values of actual measurements, the inhibition by eserine seems to begin with the binding of one

molecule of eserine with one molecule of enzyme and the process of inhibition may thus be represented as follows:

$$E+G \underset{}{\overset{Kg}{\rightleftarrows}} EG \quad \text{..... (Eq. 15)}$$

$$ES+G \overset{Kg'}{\rightleftarrows} ESG \quad \text{..... (Eq. 15')}$$

Therefore, the inhibition by eserine seems to be subject to either Eq. 15 or Eq. 15', or to both.

On the assumption that the inhibition by eserine is subject to Eq. 15, [ES] will be represented as follows:

$$[ES]=\frac{[\Sigma E]}{1+Km/[S]+[S]/K'm+[G]Km/Kg[S]}$$

where Kg is the dissociation constant of Eq. 15.

Because $vg=k_3[ES]$,

$$vg=\frac{k_3[\Sigma E]}{1+Km/[S]+[S]/K'm+[G]Km/Kg[S]} \quad \text{(Eq. 16)}$$

If [G]=O,

$$v_0=\frac{k_3[\Sigma E]}{1+Km/[S]+[S]/K'm} \quad \text{..... (Eq. 17)}$$

Consequently, H will be represented as follows through the equations Eq. 13, Eq. 16 and Eq. 17,

$$H=\frac{[G]}{Kg(1+[S]/Km+[S]^2/Km\cdot K'm)+[G]} \quad \text{(Eq. 18)}$$

It will then be acknowledged that $\phi$ in Eq. 14 is equal to $Kg(1+[S]/Km+[S]^2/Km\cdot K'm)$.

Through the relationship thereof, Kg will be found as follows:

$$Kg=\frac{\phi\cdot Km}{Km+[S]+[S]^2/K'm} \quad \text{..... (Eq. 19)}$$

If the inhibition by eserine is subject to Eq. 15', the dissociation constant of Eq. 15', Kg', will be

$$Kg'=\frac{\phi\cdot[S]}{Km+[S]+[S]^2/K'm} \quad \text{..... (Eq. '19)}$$

Though the possibility is not excluded that the inhibition by eserine is expressed by both stages of Eq. 15 and Eq. 15', according to the possible assumption that the inhibition is subject to either Eq. 15 or Eq. 15', the values of Kg and Kg' for the erythrocyte cholinesterase were calculated through Eq. 19 and Eq. 19', taking the values of Km and K'm as determined above and applying the value of $\phi$ as indicated in Table I.

Kg and Kg' of erythrocyte cholinesterase are thus given in Table II.

TABLE II

*The Values of Kg and Kg′ for the Erythrocyte Cholinesterase*

| Molarity of substrate | 0.01 | 0.005 | 0.0025 | 0.001 | Average |
|---|---|---|---|---|---|
| Kg | $0.65\times10^{-8}$ | $0.76\times10^{-8}$ | $1.0\times10^{-8}$ | $1.1\times10^{-8}$ | $0.88\times10^{-8}$ |
| Kg′ | $1.76\times10^{-7}$ | $1.03\times10^{-7}$ | $0.67\times10^{-7}$ | $0.31\times10^{-7}$ | —— |

In the serum cholinesterase, the value of K′m seems to be large enough and consequently $[S]^2/K'm$ may be accounted as negligible, and the values of Kg and Kg′ are calculated as follows:

TABLE III

*The Values of Kg and Kg′ for the Serum Cholinesterase*

| Molarity. of substrate | 0.02 | 0.01 | 0.005 | Average |
|---|---|---|---|---|
| Kg | $0.84\times10^{-8}$ | $0.87\times10^{-8}$ | $1.04\times10^{-8}$ | $0.91\times10^{-8}$ |
| Kg′ | $1.49\times10^{-7}$ | $0.73\times10^{-7}$ | $0.43\times10^{-7}$ | —— |

Thus, in both cholinesterase, the values of Kg were obtained as constant but those of Kg′ were not. Consequently, though the inhibition expressed by Eq. 15′ may not yet be negligible, that expressed by Eq. 15 seems to dominate. Eserine is thus realized to inhibit the reaction of Eq. 1. It can, therefore, be assumed that the inhibition of both erythrocyte and serum cholinesterase by eserine may be brought about in just the same way.

The fact that the values of Kg of erythrocyte and serum cholinesterase were obtained as approximately equal will introduce the assumption that at least one of the two active groups (substituting groups) in both enzymes should be the same.

SUMMARY

1. As to the human blood cholinesterase, the relationship between enzymes and substrate was analyzed from a kinetic point of view, in

order to explain the presentation of difference in erythrocyte and serum cholinesterase which have been expressed by the pS-activity curve.

2. Michaelis constants for erythrocyte and serum cholinesterase has been calculated as $3.7 \times 10^{-4}$mole/lit. and $1.2 \times 10^{-3}$mole/lit. respectively.

3. The inhibition by excess of substrate is subject to formation of ESS inactive complex produced by the combination of one molecule of enzyme with two molecules of substrate. The dissociation constant of ESS for erythrocyte cholinesterase was calculated as $1.2 \times 10^{-2}$mole/*l*.

4. The inhibition by eserine is caused by the combination of one molecule of eserine with one molecule of enzyme, and the dissociation constants for erythrocyte and serum cholinesterase-eserine complexes were calculated as $0.88 \times 10^{-8}$mole/lit. and $0.91 \times 10^{-8}$mole/lit. respectively.

5. The inhibition of erythrocyte and serum cholinesterase by eserine is subject fundamentally to the same process.

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# CARBOHYDRATE METABOLISM OF TRICHOMONAS FOETUS

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In 1928 Riedmüller discovered *Trichomonas foetus*, a polyflagellated protozoa which is parasitic in the cow vagina and causes some reproductive defects. Witte succeeded in cultivating this protozoa under a bacteria-free state in a medium consisting of peptone, broth and serum. This suggested that serum contained some essential growth factors. Many other investigators have tried to culture the microorganism in a synthetic medium, but without success. Cailleau (1934) (*1*) and Riedmüller (1936) (*2*) studied on the utilization of various carbohydrates as carbon source by *T. foetus*. They observed that a marked pH reduction of the medium was brought about by the development of the parasite. J. Andrews and T. von Brand (1938) (*3*) carried out some quantitative studies on the glucose consumption by *T. foetus* and found that a gas was produced by *T. foetus* growing in serum broth with 0.1% glucose, which burned explosively when mixed with air. Except the simple observation reported by Willens *et al.* (1942) (*4*) on the aerobic carbohydrate metabolism of *T. hepatica*, no biochemical study has been made on the metabolism of any trichomonads.

The present authors, with the object of approaching rationally to an ideal anti-trichomonad drug, and with biochemical interest, especially from the view point of comparative biochemistry, have made some studies on its carbohydrate metabolism. The present paper describes the results obtained.

## EXPERIMENTAL

### METHODS

*The culture of T. foetus and the preparation of the suspension*—The parasite was cultivated at 37° for 48 hours on boiled beef extract containing 1% peptone, 10% bovine serum, 1% glucose and 0.5% NaCl and separated

by gentle centrifugation. After being washed twice with a 0.25 *M* NaCl soultion, it was resuspended in a solution containing salts as follows: NaCl, 0.138 *M*; KCl, 0.050 *M*; $MgCl_2$, 0.005 *M*; phosphate buffer (pH 7.6), 0.01 *M*.

*Manometry and analytical methods*—Manometric measurements were made of oxygen uptake, of $CO_2$ evolution and of anaerobic acid formation with the Warburg apparatus as described by Umbreit (*5*). Lactic acid was determined by the method of Barker and Summerson (*6*) and pyruvic acid by the Friedemann and Haugen's method as modified by T. Shimidu (*7*). The determination of glucose was carried out by the Hagedorn-Jensen's method or the colorimetric method of Park and Johnson (*5*). Succinic acid was determined enzymatically (*5*). Micro gas analysis was carried out by use of the apparatus designed by C. Koyama (*8*).

## RESULTS AND DISCUSSIONS

I. Preliminary test for determining the standard conditions for the study of glucose oxidation.

Taking into consideration that this protozoa, parasitic in nature, is more sensitive to variation in external conditions, such as pH, salts concentration, temperature, *etc.*, than ordinary bacteria, we made preliminary tests as follows:

*The Influences of Saline Concentration on the Oxygen Uptake by T. foetus.*—As the parasite is greatly affected by the osmotic pressure, in the first place study was made of the effect of NaCl concentration in the suspension on the respiration. The optimal saline concentration was found to be 0.19–0.26 *M*. Outside the range of 0.35—0.1 *M* saline concentration the parasite was deformed by the osmotic pressure or swelling, and rapidly killed, and no oxygen uptake was observed. (Fig. 1 a)

*The Effect of pH*—The effect of pH of medium on the oxygen uptake was indicated in Fig. 1 b, *i. e.*, the initial pH range of 7.0—7.6 was optimal.

*The Relation among Oxygen Uptake and Glucose Concentration and the Number of the Parasite*—When the suspension containing $1 \times 10^7$ parasites per one ml. was used, the glucose concentration higher than 0.01 *M* was no more a limiting factor on the rate of oxygen uptake. When the number of the parasite per one ml. of the suspension was varied from

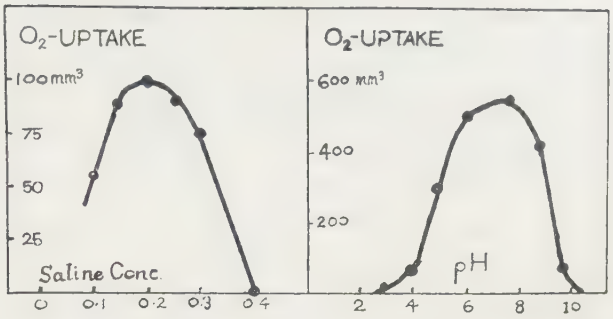



FIG. 1a. FIG. 1b.

FIG. 1a and 1b. The effect of saline concentration and pH upon the oxygen uptake by *T. foetus*.

$0.75 \times 10^7$ to $3.0 \times 10^7$, the oxygen uptake calculated as cu. mm. of oxygen per $10^7$ parasites under the standard condition remained constant below $1 \times 10^7$, while the thicker suspension showed a rapidly declining rate of oxygen uptake. Use of the suspension containing about $10^7$ parasites was most suitable for studies. The dry weight of $10^7$ parasites was about 10 mg.

*Preliminary Tests on Gas Exchange under both Aerobic and Anaerobic Conditions*—First of all, we preliminarily measured the oxygen uptake and acid formation under anaerobic condition, using glucose as substrate. (Fig. 2) The $Q_{O_2}^{air}$ value in the absence and presence of glucose was 9–10, and 15–24, respectively. The rate of the oxygen uptake was increased by 10% when the gas phase was replaced by pure oxygen. The $Q_{\text{total acid}}^{N_2-CO_2}$ value, namely the acid formation under anaerobic condition ($N_2$ 95%, $CO_2$ 5%) was 9–10 (endogeneous) and 30–45 (in the presence of glucose). These results suggested that in the trichomonad the anaerobic metabolism was predominant over the oxidative metabolism. An attempt was made to obtain the "resting" state devoid of the endogeneous respiration which was rather high, but in vain. It was porbably due to the difficulty in removing a larger quantity of the reserved substances in the parasite than in ordinary bacteria.

J. Andrews and T. von Brand (*3*) reported that a gas other than $CO_2$ was produced in the course of the growth, so we should ascertain whether or not in the case of our manometric study the gas evolved is exclusively $O_2$ and $CO_2$. Then the evolution of a gas other than $CO_2$ was measured under pure nitrogen as atmosphere with the flask 1 with

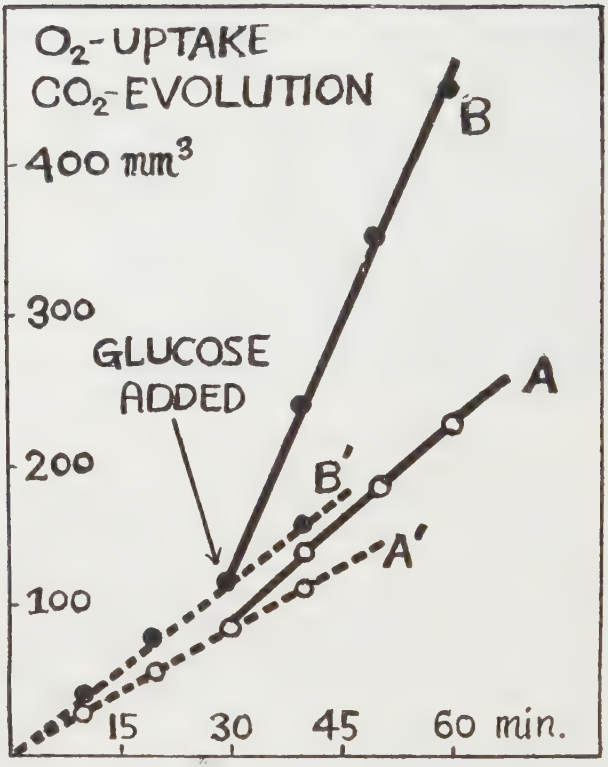



FIG. 2. Oxygen uptake and anaerobic acid formation with or without glucose by *T. foetus* suspension.

A : in the presence of glucose
A′: endogeneous
} oxygen uptake

B: in the presence of glucose
B′: endogeneous.
} $CO_2$-evolution under anaerobic $CO_2$-bicarbonate condition.

KOH in the centre well and the total gas evolved, by the flask 2 without KOH in it. 1′ and 2′ corresponded respectively to 1 and 2 without glucose as substrate. As evident from Fig. 3, under the anaerobic condition not only $CO_2$ but also another unknown gas were produced. When nitrogen was replaced by air after 120 minutes, the vigorous oxygen uptake was observed in flask 1. Even if the same unknown gas was involved in the aerobic condition, its amount would be so small as to be negligible compared with the rate of oxygen uptake, judging from the

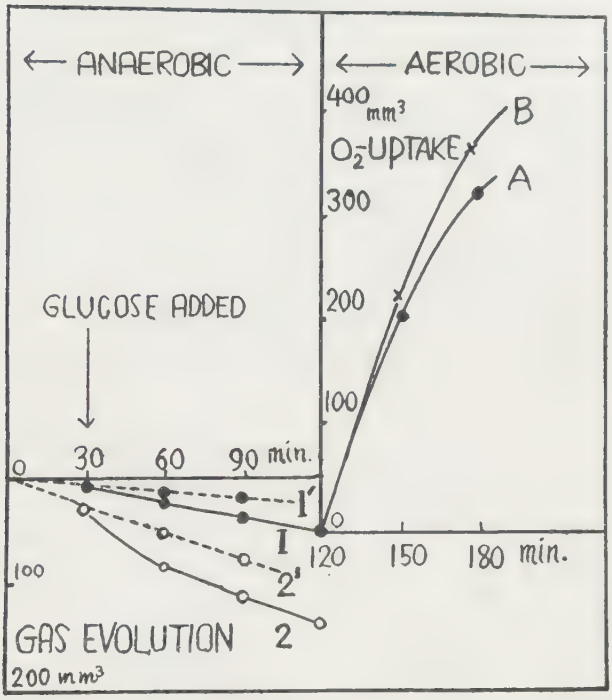



FIG. 3. The preliminary test for the manometric study of *T. foetus* suspension: gas metabolism.

Under anaerobic condition: Gas evolution.
1 : KOH present, with glucose.
1′: KOH present, endogeneous.
2 : KOH absent, with glucose.
2′: KOH absent, endogeneous.
Under aerobic condition: Oxygen uptake.
A : In the atmosphere of air.
B: In the atmosphere of pure oxygen.

fact that the former is really one-fifteenth as much as the latter in the anaerobic condition.

II. The respiration of *T. foetus*.

*The Effects of Various Substrates on the Respiration*—To test the ability of *T. foetus* to oxidize various substrates, we measured manometrically the respective oxygen uptake in the presence of each substtate (0.1—

0.2 *M* in final concentration) Of 27 substrates tested, glucose, maltose and sucrose were rapidly, for the most part, oxidized; fructose, mannose and galactose were oxidized at half so much a rate as glucose, while the other substrates were oxidized very slowly or not at all. It is surprising to note that zymohexose and its related disaccharides were oxidized while the other substrates, that is, $C_3$-compounds, $C_4$-dicarboxylic acids, lower fatty acids and some amino acids, were not oxidized at all. (Tab. I). Another experiments showed that the oxidation of glucose was not activated by the addition of $C_4$-dicarboxylic acids, such as succinate, fumarate and malate, and that the addition of $C_4$-dicarboxylic acids could not give the effect on the oxidation of pyruvate or lactate.

*The Effects of Various Enzyme Inhibitors on the Oxygen Uptake*—The effect of some usual enzyme inhibitors on the respiration of *T. foetus* with glucose as substrate are shown in Fig. 4, illustrating the relation

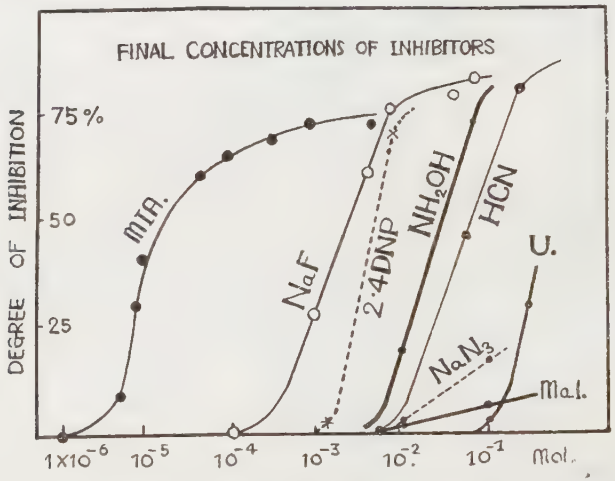



FIG. 4. The relation between the degree of inhibition (%) and the malar concentration of each inhibitor.

MIA: Monoiodoacetic acid; 2,4-DNP: 2,4-Dinitrophenol; U: Urethane; Mal: Malonate.

between the concentration and the rate of inhibition. It is noticeable from these data that (a) iron porphyrine enzyme poisons, such as cyanide, azide and hydroxylamine, as well as malonate, a well-known inhibitor of succinic dehydrogenase, not inhibit the respiration, and that (b) mono-iodoacetate and fluoride, the inhibition of which are character-

istic of the Embden-Meyerhof-Parnas' glycolytic process, show a marked inhibition even in the lower concentrations (0.00005 *M*, and 0.001 *M*, respectively).

*The Aerobic Breakdown of Glucose*—Although the endogeneous respiration of *T. foetus* was rather high, since it maintained the constant rate

TABLE I

*The Effect of Various Substrates on the Oxygen Uptake by T. foetus Suspension*

| Substrates | Final concentration | Oxygen uptake* 30 min. | 60 min. | Difference between** each value and the endogeneous $O_2$-uptake |
|---|---|---|---|---|
| Endgeneous | | 53 | 100 | |
| Xylose | 0.02 *M* | 50 | 101 | 1 |
| Arabinose | 0.02 *M* | 52 | 153 | 3 |
| Glucose | 0.02 *M* | 124 | 235 | 100 |
| Fructose | 0.02 *M* | 102 | 221 | 89 |
| Sorbose | 0.02 *M* | 56 | 104 | 4 |
| Mannose | 0.02 *M* | 89 | 185 | 63 |
| Galactose | 0.02 *M* | 92 | 178 | 58 |
| Sucrose | 0.02 *M* | 113 | 247 | 109 |
| Maltose | 0.02 *M* | 117 | 236 | 100 |
| Lactose | 0.02 *M* | 54 | 125 | 19 |
| Dextrine | 0.2 % | 62 | 125 | 19 |
| Soluble starch | 0.2 % | 55 | 112 | 9 |
| Glycogen | 0.2 % | 54 | 110 | 7 |
| Glycerol | 0.02 *M* | 72 | 146 | 34 |
| Ethanol | 0.02 *M* | 56 | 109 | 7 |
| Lactate | 0.02 *M* | 56 | 108 | 6 |
| Pyruvate | 0.02 *M* | 55 | 113 | 10 |
| Fumarate | 0.02 *M* | 52 | 109 | 7 |
| Succinate | 0.02 *M* | 48 | 98 | −2 |
| Malate | 0.02 *M* | 50 | 113 | 10 |
| Citrate | 0.02 *M* | 56 | 102 | 2 |
| Formate | 0.02 *M* | 64 | 123 | 17 |
| Acetate | 0.02 *M* | 54 | 106 | 4 |
| Glycine | 0.02 *M* | 55 | 112 | 9 |
| DL-α-Alanine | 0.02 *M* | 52 | 107 | 5 |
| DL-β-Alanine | 0.02 *M* | 51 | 100 | 0 |
| L-Aspartate | 0.013 *M* | 51 | 102 | 0 |
| L-Glutamate | 0.013 *M* | 48 | 98 | −2 |
| L-Tyrosine | saturated | 50 | 100 | 0 |

* Each relative value is calculated on the basis of the endogeneous oxygen uptake in 60 minutes as 100.

** Each is on the basis of glucose as 100.

of the oxygen uptake for several hours, the corresponding difference between the oxygen uptake without (endogeneous) and with glucose as substrate reached a certain value after about two hours, as shown by Fig. 5. In this case, one molecule of glucose seemed to have consumed

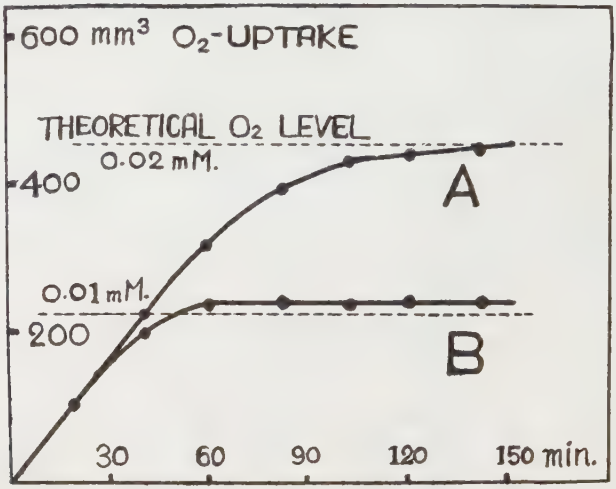



FIG. 5. The relation between the amount of glucose added and oxygen uptake.

A: 20 micromoles glucose added.

B: 10 micromoles glucose added.

one molecule of oxygen to form one equivalent acidic group. The acid formed was not yet identified (Tab. II).

Cailleau (*T. foetus*, *T. columbae*) (1934) (*1*), Riedmüller (*T. foetus*) (1936) (*2*), and Trussel and Johnson (*T. vaginalis*) (1941) (*3*) investigated the utilization of various carbohydrates as carbon source in the cultivation of the respective trichomonad. As for *T. foetus*, it utilized

TABLE II

*The Oxidative Bbreakdown of Glucose and the Acid Formation*

| Glucose added *micromoles* | $O_2$-uptake *cu.mm.* | Acid formed *micromoles** | Molar ratio $O_2$/glucose | Molar ratio acid/$O_2$ |
|---|---|---|---|---|
| 10 | 240 | 8 | 1.07 | 0.80 |
| 20 | 440 | 17 | 0.98 | 0.85 |

* The acid formed was titrated with $N/100$ NaOH and calculated as monobasic acid.

zymohexose, galactose, lactose, maltose, dextrine, starch and glycogen but not tetroses, pentoses and polyalcohols. Our manometric results were almost consistent with their cultivating results, except that lactose and polysaccharides were not utilized in the respiration.

Neither lactate nor pyruvate was aerobically oxidized by *T. foetus*. $C_4$-dicarboxylic acids and citric acid did not also activate the respiration with or without glucose. Malonate did not inhibit the oxygen uptake. Considering these results, it is unlikely that the Krebs' tricarboxylic acid cycle takes part in the cell metabolism of *T. foetus*. The facts that the respiration of *T. foetus* was to a great extent inhibited by the glycolytic poisons (monoiodoacetate, fluoride, *etc.*), but not by cyanide, azide, *etc.*, and that acid formation took place even under the aerobic condition, suggested that the metabolism of this organism has some bearing to the Embden-Meyerhof-Parnas' glycolytic process.

The inability of cyanide and of azide to inhibit the oxygen uptake gave the question, whether cytochromes and cytochrome oxidase system existed in this organism. Spectroscopic observation revealed two distinct absorption bands at 565 and 535 m$\mu$, which were identified as cytochrome b, but the bands corresponding to cytochrome a and c and to cytochrome oxidase were not found. It is interesting that we observed in *T. foetus* the presence of cytochrome b, which is not one of the *E. coli* type, but one of the muscle type. Namely, the latter is autoxidizable and insensitive to cyanide, while the former is considered by F. Egami *et al.* (10) to be nitrate reductase. *T. foetus* suspension had no ability to reduce nitrate to nitrite. As for other heme enzymes, the existence of catalase in water extract of minced *T. foetus* was also ascertained by both titrimetry and manometry and that of peroxidase by purpurogallin test.

The study on *T. hepatica* made by Willems, Massart and Peeters (*4*) (1942) is the first and only investigation hitherto made on the metabolism of any trichomonad. Their results with *T. hepatica* almost coincides with our results on *T. foetus* with respect to the effects of substrates and enzyme inhibitors on the respiration. On the basis of these results the organism seems to be losing progressively its power of metabolizing glucose aerobically, due to the long-continued parasitic life.

III. Anaerobic metabolism of glucose.

Preliminary experiments led us to anticipate that the acid formation during the course of the anaerobic breakdown of glucose by *T. foetus*

was related to the usual glycolytic process. Therefore, we made a further study on this point.

*The Anaerobic Breakdown of Glucose and the Acid Formation*—Using the suspension containing 0.025 *M* $NaHCO_3$ under the anaerobic gas mixture (95% $N_2$, 5% $CO_2$), we measured the total gas evolution in the presence of glucose. As shown in Fig. 6, the total gas evolution was proportional

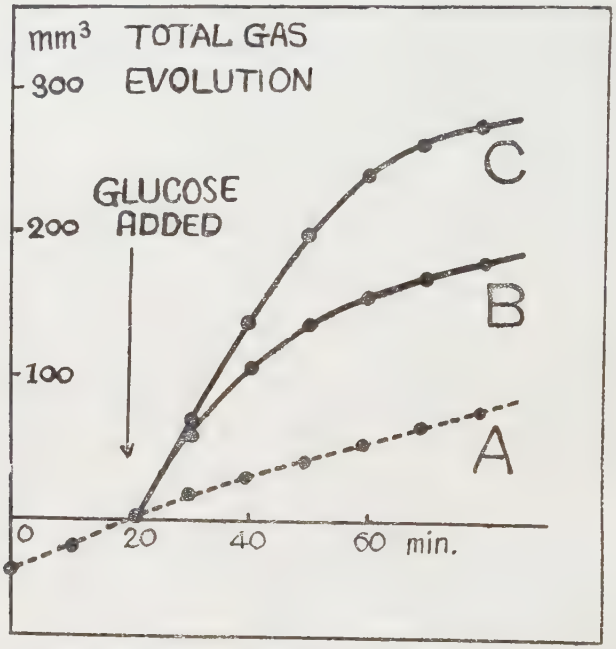



FIG. 6. The acid formation from glucose under anaerobic condition by *T. foetus* suspension.

A: Endogeneous.
B: 5 micromoles glucose added.
C: 10 micromoles glucose added.

to the amount of glucose added. Although it seemed to behave as if one acidic group was produced from one molecule of glucose, it was not strictly the case, because this total gas evolved contained not only $CO_2$ formed by the neutralization of bicarbonate by the acid formed, but also the metabolic $CO_2$ and the unknown gas. Determination of glucose, pyruvate and lactate showed that the amounts of pyruvic and

lactic acids formed were 1/10 times less than that of glucose consumed (Tab. III). Namely, the main end product from glucose was neither lactic nor pyruvic acid, which was found in the ordinary glycolytic process.

TABLE III

*The Anaerobic Breakdown of Glucose and Acid Formation (1)*

| | Pyruvic acid | Lactic acid | Glucose | Total gas evolved |
|---|---|---|---|---|
| *min.* | *micromoles* | *mibromoles* | *micromoles* | *cu.mm.* |
| t=0 endogeneous | 0.04 | 0 | — | — |
| t=0 glucose | 0.04 | 0 | 41.5 | — |
| t=60 endogeneous | 0.04 | 0 | 1.5 | 98 |
| t=60 glucose | 0.39 | 0.84 | 25.3 | 507 |
| The differences in 60 min. | +0.35 | +0.84 | −16.2 | +409 |
| Molar ratios | pyruvic/glucose: 0.02, lactic/glucose: 0.05, gas/glucose: 1.1 | | | |

Then, we tried to investigate more exactly this glucose decomposition. We measured succinic acid enzymatically and the total acid production by determining the amount of the remained bicarbonate. As shown in Tab. IV, about 70% of the total acids formed was found to be succinic acid. When glycerol or hexose diphosphate was added in place of glucose under the same condition, no acid formation occurred.

TABLE IV

*The Anaerobic Breakdown of Glucose and Acid Formation (2)*

| | t=0 | t=30 min. | Difference |
|---|---|---|---|
| Glucose, present *micromoles* | 11.50 | 6.25 | −5.25 |
| Bicarbonate, remained *micromoles* | 25.00 | 19.50 | −5.50 |
| Succinic acid, present *micromoles* | 0.300 | 2.30 | +2.00 |

The amount of succinic acid formed in 30 minutes was 73% of the total acids formed, since it was a dicarboxylic acid.

*The Evolution of Hydrogen*—An investigation on the anaerobic glucose metabolism was further made in order to differentiate the metaboic $CO_2$, the unknown gas evolved and acid formation. For this purpose

three flasks were used, of which both flask I containing 20% KOH in the center well and flask II without KOH were filled with nitrogen as gas phase, and flask III was kept in a condition of an anaerobic carbon dioxide—bicarbonate buffer. We discerned an evolved gas other than $CO_2$ from I and estimated the metabolic $CO_2$ evolution by subtracting I from II, and acid formation by substracting II from III. Fig. 7 illus-

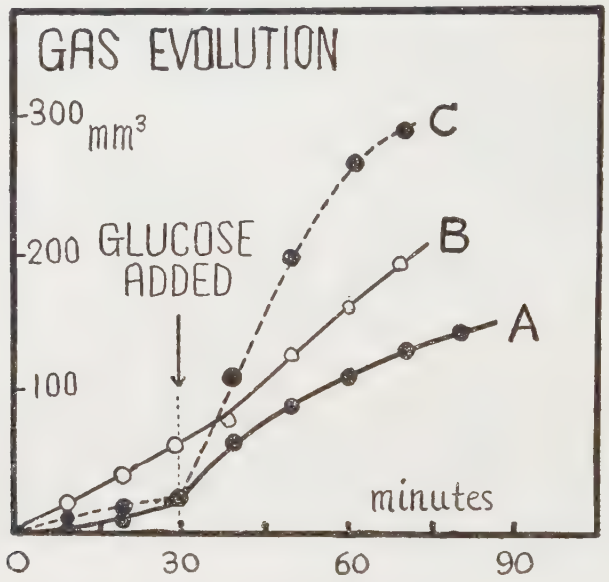



FIG. 7. The relation of the evolution of the metabolic $CO_2$ and of hydrogen and the acid formation in the course of the anaerobic glucose breakdown by *T. foetus* suspension.

Under the atmosphere of pure nitrogen,

Flask I: KOH present in the center well. Hydrogen evolution.

Flask II: KOH absent. $CO_2$ and $H_2$ evolution.

Under the atmosphere of 95% $N_2$, 5% $CO_2$, in the bicarbonate medium.

Flask III: Total gas evolution.

A: Hydrogen evolution: (Flask I).

B: The metabolic $CO_2$ evolution; (II)-(I).

C: $CO_2$ production by acid formation; (III)-(II).

trated this experimental results, showing clearly that not only the metabolic $CO_2$ but an unknown gas evolved under this condition. An

important problem is to identify this unknown gas, but we have no available manometrical method for this purpose. However, we can give an evidence sufficient and reliable in the culture experiment to show that the gas in question is hydrogen. The evolution of the metabolic $CO_2$ was scarcely increased by the addition of glucose, while both acid formation and hydrogen evolution depended on the amount of glucose added and not on that of peptone. (Fig. 8 and Tab. V) These facts

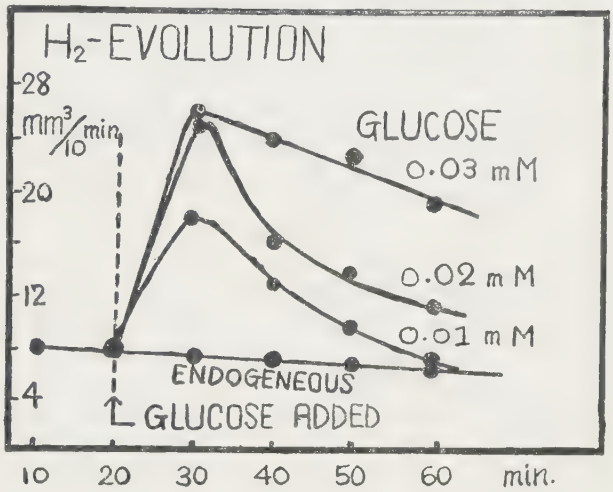



FIG. 8. The relation between the hydrogen evolution and the amount of glucose added.

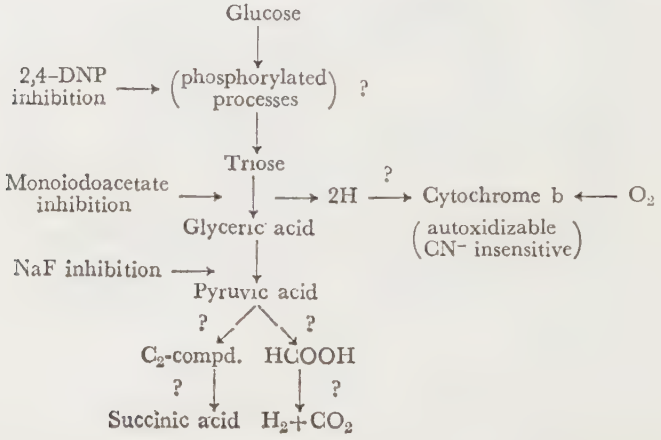
TABLE V

*The Effect of Some Substrates and pH on the Hydrogen Evolution*

| Substrate | Final concentration | pH | The amount of hydrogen evolved in 30 min.* |
|---|---|---|---|
| Endogeneous | — | 7.0 | 100 |
| Glucose | 0.01 *M* | 4.8 | 133 |
| | 0.01 *M* | 7.0 | 234 |
| | 0.01 *M* | 8.5 | 264 |
| Peptone | 1 % | 7.0 | 99 |
| Pyruvate | 0.01 *M* | 7.0 | 150 |
| Formate | 0.07 *M* | 7.0 | 128 |

* Each relative values, on the basis of the endogeneous evolution as 100.

showed that this hydrogen evolution was connected with the carbohydrate metabolism, but the rate of hydrogen evolution was not activated by the addition of glycerol, lactate or DL-$\alpha$-alanine. On the other hand, hydrogen evolution was somewhat increased by the addition of pyruvate or formate. (Tab. V) By means of the Thunberg's decolorising test with methylene blue, we found the rather powerful activity of formic dehydrogenase in *T. foetus*, but could obtain no evidence for the existence of hydrogenlyase or hydrogenase, to which we might attribute such a hydrogen evolution. In consideration of these results, the anaerobic breakdown of glucose by *T. foetus* differs considerably from the usual glycolytic process. In conclusion, we should like to propose the following scheme of glucose breakdown. The scheme explains our observations on the intact parasite very well. It is, however, desirable and necessary to extend our observation to intermediates of the Embden-Meyerhof scheme by




using the homogenates or the acetone-powders. We should have ascertained the direct evidences that (I) hydrogen is transferred through cytochrome b to oxygen consumed and (II) the mechanisms of hydrogen evolution and of succinic acid formation are carried out in accordance with the metabolic sequence of the scheme above. It is very important to investigate the enzyme systems involved in this new type of glucose decomposition to succinic acid accompanied by hydrogen evolution.

Judging from the viewpoint of the comparative biochemistry with the parasitic protozoon, the carbohydrate metabolism of *T. foetus* seems to bear rather striking similarities to that of the African pathogenic

trypanosomes. T. von Brand *et al* divided mammalian trypanosomes into two groups accoding to the effect of cyanide on their respiration (*11, 12*). The respiration of the lewisi subgroups is sensitive to cyanide, while that of the evansi subgroups is unaffected or even stimulated by cyanide. This classification coincides with the two groups of trypanosomes established on the basis of differences in life cycle, morphology and pathogenicity. It suggests that the trypanosomes of the evansi subgroups have evolved from their insect-inhabiting ancestors through the loss of the original heavy metal enzymes. (*13*) In addition, they are characteristic of the loss of TCA cycle, of which was well reviewed by T. von Brand. (*14*) The metabolic similarities between *T. foetus* and the pathogenic trypanosomes of the evansi subgroups (especially *Tryp. hippicum* (*15*) seem to us to throw light on the research of potential antitrichomonad drugs.

IV. Some observations on the anaerobic cultivation of *T. foetus*.

*Some Biochemical Changes of the Broth during the Cultivation*—We carried out the cultivation of *T. foetus* anaerobically in the 200 ml.-injection syringe with a cock attached to its nozzle, to push out at will an adequate amount of the culture medium or the evolved gas. We took samples in such a way and determined glucose consumed therein, lactic, pyruvic and succinic acids formed, and measured the changes of pH, the number of the parasite and the titrable acids formed. (Fig. 9) The more

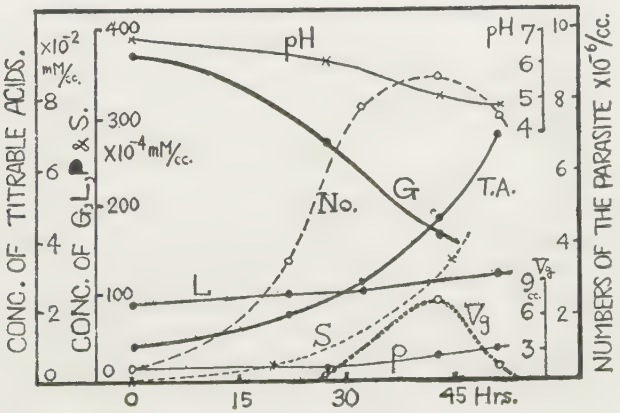



FIG. 9. Some changes occurred during the course of the development of *T. foetus* in a medium containing peptone, broth, serum and glucose.

G,L,P and S represent the concentrations of reducing sugar, lactate, pyruvate and succinate, respectively. T. A.: titrable acids, formed during the cultivation; No.: numbers of the parasite; Vg.: the volume of gas produced.

was glucose consumed, the more were the titrable acids produced, when the parasite was developing. Of the total amount of titrable acids produced in 44 hours, that of succinic acid was 83%, but that of lactic and pyruvic acids, less than 10%, respectively. In order to obtain the end products, 500 ml. of broth were extracted with ether by the Soxlet apparatus for 120 hours. The ether was distilled off and from the residue about 800 mg. of crystalline succinic acid were obtained. Neither volatile acid nor other acid was found in an appreciable amount.

*The Gas Analyses*—By the micro gas analysis we analyzed the gas produced in an amount of 9 to 11 ml. from the 50 ml. culture medium and found that the gas contained a large quantity of hydrogen and a small quantity of methane. (Tab. VI) This fact confirmed the observation of J. Andrews and T. von Brand (*3*) that the gas burned explosively when mixed with air and gave the evidence that the unknown gas we measured manometrically other than $CO_2$ was most probably hydrogen.

TABLE IV

*The Composition of Gas Evolved during the Anaerobic Cultivation*

| Exp. No. | Vol. of gas evolved/50 ml. medium, | $CO_2$ | $O_2$ | $CH_4$ | $H_2$ | $N_2$ |
|---|---|---|---|---|---|---|
| 1 | 10.0 *ml.* | 0.00 % | 1.77 % | 1.89 % | 83.73 % | 12.61 % |
| 3 | 9.4 | 0.00 | 6.00 | 0.30 | 73.65 | 20.04 |
| 7 | 11.9 | 1.96 | 5.57 | 2.05 | 78.65 | 11.77 |

SUMMARY

Using the suspension of *Trichomonas foetas*, one of the polyflagellated protozoa, the carbohydrate metabolism was studied manometrically under both aerobic and anaerobic conditions and the biochemical changes during the course of the anaerobic culture were observed.

1. The preliminary tests were carried out in order to determine the standard conditions for the study of glucose oxidation, that is, with respect to the effects of pH, salt concentration, glucose concentration and the numbers of the parasite on the oxygen uptake of *T. foetus*.

2. Of the twenty seven substrates tested, only glucose, fructose, mannose, galactose, sucrose and maltose increased the oxygen uptake of the parasite, while the others did not evidently activate the endogene-

ous respiration. Especially, lactate, pyruvate and $C_4$-dicarboxylic acids did not influence the respiration of the parasite, and malonate had no inhibitory effect on the oxygen uptake. These facts suggested that Krebs' tricarboxylic acid cycle did not take part in this aerobic carbohydrate metabolism.

3. The facts that the respiration of this protozoon was strongly inhibited by monoiodoacetate (0.00005 *M*) and sodium fluoride (0.001 *M*), but not by cyanide (0.01 *M*) and azide (0.1 *M*) and that acid formation was observed in the aerobic breakdown of glucose, gave the suggestion that the Embden-Meyerhof-Parnas' glycolytic process has relation to this glucose metabolism.

We demonstrated the presence of catalase and peroxidase in minced juice of the parasites and also ascertained spectroscopically the existence of cytochrome b in *T. foetus*, but could find neither the absorption bands of cytochrome a and c nor that of cytochrome oxidase.

4. In the anaerobic breakdown of glucose, the parasite evolved hydrogen gas as well as metabolic carbon dioxide, and produced a large quantity of acids, of which more than 70% was identified as succinic acid. Both lactic and pyruvic acids were scarcely produced in the same condition. In these facts, this anaerobic glucose metabolism differed considerably from the usual glycolysis.

5. During the course of the anaerobic cultivation of this protozoon, the titrable acids formation proceeded in parallel with glucose consumption, while the parasites were developing. Of the total amount of the titrable acids, that of succinic acid was 83%, but that of lactic and pyruvic acids, less than 10%. The gas produced in the cultivation was shown by micro gas analysis to contain a large amount of hydrogen and a small amount of methane.

We are deeply indebted to Prof. K. Okunuki for the spectroscopic observation and to Dr. C. Koyama for micro gas analyses and to Mr. H. Huzitani for the cultivation of the parasite. The authors' thanks are also due to Prof. F. Egami for his continued interest and advice.

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# ON THE CAUSE OF THE S-SHAPED RATE-LIGHT INTENSITY-RELATIONSHIP IN THE PHOTOSYNTHESIS OF PURPLE BACTERIA

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Since the observation by French (*1*) in 1937 it has been known that the photosynthetic process of purple bacteria shows a peculiar phenomenon in the dependency of its apparent rate upon the light intensity. On plotting the rate against the light intensity, an S-shaped curve was obtained instead of a hyperbolic one as is the case with green algae (*6, 7*). It had been conjectured that this phenomenon may be caused by some intracellular hydrogen-donor (French (*2*), Wassink (*9, 10*)), but sofar no satisfactory explanation, backed by sufficient experimental evidence, has yet been forthcoming.

In this paper experiments are described which allow us to conclude that the phenomenon in question is due to a process of photochemical hydrogen evolution which occurs concomitantly with the normal photosynthesis of bacteria. It will be shown that if the effect of this hydrogen evolution was eliminated by placing palladium asbestos in manometer vessels, the rate-light intensity-relationship becomes quite similar to that observed with green plants. It was further ascertained that under such conditions the dark reaction of bacterial photosynthesis shows a rate-temperature-relationship which also is essentially the same as that observed with green plants.

## MATERIAL AND METHOD

The organism used was a typical nonsulfur purple bacterium, *Rhodobacillus palustris* Molisch. Cultures were made in the Rue's flasks containing 500 ml. nutrient medium of the following constitution: sodium lactate 5 g.; pepton 5 g.; pond water 1000 ml. (pH 7.0).

After 3 or 4 days culture at 25° under continuous illumination with a 60 watt lamp at a distance of about 30 cm., the bacteria were collected by centrifugation, washed several times with distilled water, and sus-

pended in $M/15$ phosphate buffer of pH 7.0. The rate of gas exchange was measured by the Warburg manometer with 10 ml. of the bacterial suspension which contained about 20 to 100 mg. dry weight of bacterial cells. As the hydrogen donor for the photosynthesis, either $H_2$ or butyrate was used. In the former case, the gas phase of the vessel was filled with a mixture of 95% $H_2$ and 5% $CO_2$, and in the latter case sodium butyrate was added to the bacterial suspension in the concentration of 0.01 $M$ and the gas phase of the vessel was filled with a mixture of 95% $N_2$ and 5% $CO_2$. In both cases a small piece of yellow phosphorus was placed in the center well of the vessel to ensure the anaerobic condition. In some experiments using butyrate as the hydrogen donor, 0.1 g. of 10% palladium asbestos was placed in the side arm of the vessel in order to absorb any hydrogen gas evolved. The asbestos used has previously been heated to red hot to ensure its hydrogen-absorbing capacity. Illumination was furnished by a 200 watt Mazda lamp which was placed beneath the glass-window at the bottom of the thermostat. Adjustment of the intensity of the incident light was effected by varying the distance of the lamp from the bottom of manometer vessel.

## RESULTS

*Positive Gas Evolution Observed at Lower Light Intensities*—It was found that the rate of gas absorption by our organism changes in peculiar manner with the change of the light intensity. In Fig. 1 are shown typical examples of our observations in which hydrogen was used as the H-donor.

As is evident from this figure, a positive gas evolution occurred at lower light intensities (about 500–1000 lux), the rate of which appeared to decrease rapidly with decrease of temperature.

At higher light intensities the rate of the net gas absorption increased with temperature, while the reverse was true at lower light intensities.* This phenomenon may be explained as being due to the occurrence of some gas-evolving process at lower light intensities, which by itself is accelerated by increase of temperature. That this process is a photochemical one is apparent from the fact that it ceased completely when light was turned off. The data illustrated in Fig. 1 suggest that the process of gas evolution seems to have larger temperature coefficient than the photosynthetic process occuring under higher illumination.

* Similar phenomenon has already been observed by French (*1*).

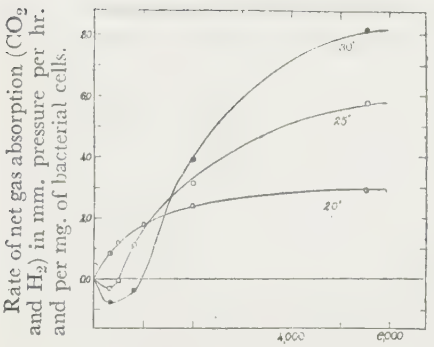



FIG. 1. Rate of the net gas absorption ($CO_2$ and $H_2$) as a function of light intensity and temperature. Hydrogen-donor: $H_2$; bacterial cells used: 26 mg.

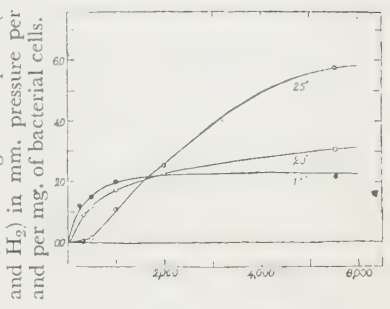
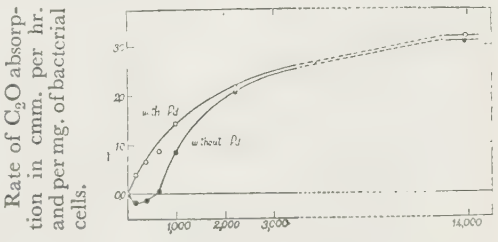



FIG. 2. Rate of the net gas absorption ($CO_2$ and $H_2$) as a function of light intensity and temperature. Hydrogen-donor: $H_2$; bacterial cells used: 25.6 mg.

Under certain culture conditions, which are not yet specified in detail, we obtained bacterial samples which did not show recognizable positive gas evolution under weak light. With these samples the rate-light intensity-curves assumed an S-form as was observed by earlier workers, and these curves were found to be influenced by temperature as shown in Fig. 2. The fact that also in this case the curves for different temperatures crossed each other at certain light intensity indicates that also in these samples the temperature-dependent process of gas evolution was occuring at lower light intensities.

When instead of $H_2$, butyrate was used as hydrogen-donor, the gas-evolving process appeared to be less marked, but its occurrence is undoubted as may be seen from the result given in Fig. 3. In this figure is also shown the effect of the presence of palladium asbestos in the manometer vessel. By the effect of palladium asbestos the dent of the




FIFIG. 3. The rate of gas exchange at varying light intensities observed with and without palladium asbestos placed in the manometer vessel. Hydrogen-donor: butyrate; Bacterial cells used: 52 mg.

curve at lower light intensities totally disappeared, and a smooth curve similar to those obtained with green plants was brought about. The fact observed in this experiment shows conclussively that the gas evolved at lower light intensities was nothing but hydrogen. It may be quite reasonable to infer that also the gas evolved in the experiments using $H_2$ as the hydrogen-donor was of the same nature.

*Effect of Various Factors upon the Process of $H_2$ Evolution*—Photochemical production of hydrogen from photosynthesizing cells has already been observed with a certain strain of *Scenedesmus* by Gaffron and Rubin (*3*) and recently with *Rhodospirillum rubrum* by Gest and Kamen (*4*, *5*). According to Gaffron *et al.*, the hydrogen production by *Scenedesmus* occurred at low light intensity after long anaerobic incubation of the cells in the absence of $CO_2$. Gest *et al.* have observed that the photoproduction of hydrogen by *Rhodospirillum* occurred without previous adaptation, but required certain exogenous substrates such as malic, fumaric, oxaloacetic, or pyruvic acids. Noticiable was the fact that the bacterium did not produce $H_2$ under nitrogen atmosphere or in the presence of ammonium ion. Based on these findings, Gest *et al.* assume that the hydrogen evolution by *Rhodospirillum* is linked to nitrogen metabolism and perhaps to a nitrogenase system, the existence of which in the same bacterium has been demonstrated by said authors. The question now arises as to whether the process we have observed has any essential bearing on the corresponding phenomena in *Scenedesmus* or *Rhodospirillum*.

As may be seen by comparison of experimental results given in Fig. 1 and 3, the rate of photoproduction of hydrogen by our organism was less marked in $N_2$-$CO_2$-atmosphere (butyrate as substrate) than in $H_2$-$CO_2$-atmosphere (hydrogen as substrate). This may indicate that the molecular nitrogen has some suppressing effect upon the process of hydrogen production, though it may as well be interpreted as being due to the unsuitableness of butyrate as a substrate of $H_2$-production. Using $H_2$ as substrate, the effect of ammonium ion (ammonium nitrate) was investigated, and it was found to retard the $H_2$-production to some extent but not completely. At any rate, neither $N_2$ nor ammonium ion could halt the $H_2$-production by our organism completely as it was the case with *Rhodospirillum*. Similarity and difference were observed also in the effect of exogenous substrates; enhancing effect was found in fumarate, succinate and pyruvate, but not in malate which was reported to be most effective in increasing the $H_2$-production by *Rhodospirillum*.

Incubation of the bacterium in anaerobic conditions (dark and

light) had no appreciable effect upon the subsequent capacity of $H_2$-production. On the other hand it was markedly depressed when the bacterium was left illuminated for several days under aerobic condition.

Depression of the activity of $H_2$-production was caused also by the following treatments: (a) pretreatment of bacterial cells with a phosphate buffer of pH 4.0 for one hour, (b) irradiation with ultraviolet light, (c) heating of bacterial cells at 45°, for 1 hour,* (d) addition of hydroxylamine to the reaction mixture. Hydroxylamine causes also the depression of the normal photosynthesis, but its effect upon the prosess of $H_2$-production was much more pronounced. This may be seen from the curves in Fig. 4 which illustrates the effect of $10^{-3}M$ hydroxlamine upon the rate of gas exchange in the presence of $H_2$ as hydrogen-donor.

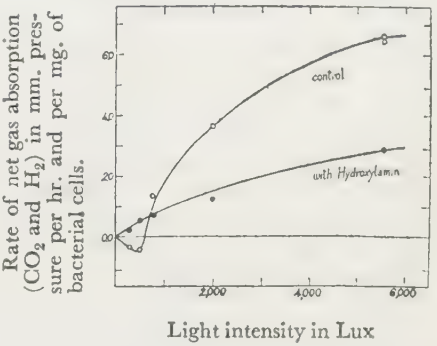



FIG. 4. Effect of hydroxylamine ($10^{-3}$ *M*) on the rate of the net gas absorption. Hydrogen-donor: $H_2$; Bacterial cells used: 25 mg.

Using $H_2$ as hydrogen-donor, it was further found that the photochemical $H_2$-production occurred more pronounced at pH 5.4 than at pH 7.0 and pH 9.4.

The observation described above show that the $H_2$-production by our organism is not quite similar, but in some respects bears a resemblances to that observed by Gest and Kamen in *Rhodospirillum*. According to Gest *et al.* the photoproduction of hydrogen by *Rhodospirillum* occurs not only in weak light but also in strong light. In our experiment the positive $H_2$-production appeared to take place only in weak light; There may, however, be a posibility that it had occurred also in stronger light, having escaped from our observation presumably, because of the occurence of the photosynthestic reaction: $2H_2+CO_2 \rightarrow HCHO+H_2O$. The question as to whether it occurs, as the $H_2$-production by *Scenedəsmus*, only under weak light is left open to further investigations.

*The Rate-Light Intensity-Relationship of the Photosynthesis Using Butyrate as a Hydrogen-Donor under the Condition of Excluding the Effect of $H_2$-Produc-*

* Noteworthy is the fact that by this treatment the proper photosynthetic process was not affected recognizably.

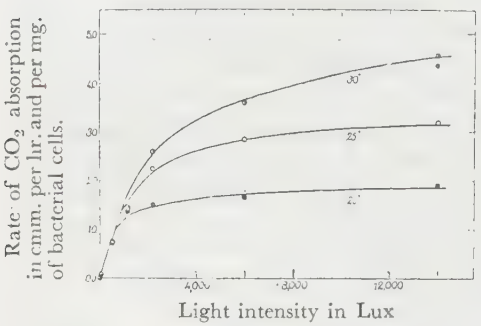



Fig. 5. The rate of gas exchange at varying light intensityies observed with palladium asbestos placed in the manometer vessel. Hydrogen-donor: butyrate; gas space: 95%$N_2$+5% $CO_2$; Bacterial cells used: 48 mg.

*tion*—Using butyrate as a hydrogen-donor and eliminating the effect of $H_2$-production by placing palladium asbestos in the manometer vessel, the rate-light intensity-relationship of bacterial photosynthesis was investigated at different temperatures. The results obtained are given in Fig. 5 from which it may be seen that the relationship is quite similar to that observed in green plants. Striking similarity to the photosynthesis of green plants was found in the following facts: (a) the rate of $CO_2$-absorption becomes temperature-independent at lower light intensities, (b) the rate-light intensity-relationship can be expressed, with good approximation, by the formula

$$V=\frac{V_m I}{I+A}$$

where $V$ is the rate of $CO_2$-absorption, $V_m$ its maximum value, $I$ the light intensity and $A$ a constant corresponding to the light intensity causing $V$ to be one half of $V_m$.

*Temperature Dependency of the Rate of the Dark Reaction Using Butyrate as Hydrogen-Donor*—It has repeatedly been shown that the temperature dependency of the dark reaction in the photosynthesis of green algae does not follow the simple Arrhenius principle. This fact has been interpreted as indicating that the dark reaction involves at least two consecutive steps having widely different temperature coefficients. Based on the data obtained with *Chlorella ellipsoidea* (under the condition of $CO_2$ saturation), Tamiya *et al* (*8*), have calculated the activation energies of these two steps to be 6 k cal. and 27 k cal., respectively. In order to make clear whether or not the similar phenomenon will be observed in the photosynthesis of purple bacterium, the following experiment was carried out.

The rate of photosynthesis of *Rhodobacillus palustris* was measured using butyrate as the hydrogen-donor under the condition which was

saturating both in respect of $CO_2$ concentration (95% $N_2$ 5% $CO_2$) and of light intensity (10,000–15,000 lux), (palladium asbestos was placed in the side arm of the vessel). Since the measurement covering wide range of temperature could not be performed with one and the same bacterial sample, the rate at different temperatures was compared with that at 17° using in each experiments different bacterial samples. The temperatures studied were 2°, 5°, 8°, 12°, 17°, 22°, 27°, and 32°. The results obtained are shown in Fig. 6 in which the logarithm of the relative rate, *i.e.*

$$\frac{\text{The rate of } CO_2 \text{ absorption at each temperature}}{\text{The rate of } CO_2 \text{ absorption at } 17°}$$

is plotted against the reciprocal of absolute temperature.

The curve given in Fig. 6 resembles strikingly those obtained for green algae by earlier workers. On the assumption that the dark reaction of bacterial photosyntnesis involves two rate-determing steps, the activation energies of these steps were computed by the analysis of the curve. The figures obtained, it is quite remarkable, were practically equal to those found for *Chlorella* by Tamiya *et al.*, namely 5–6 k cal. and 26–27 k cal., respectively. Whether these values are subjected to change according to the sort of hydrogen-donor applied, is an interesting question awaiting further investigations.

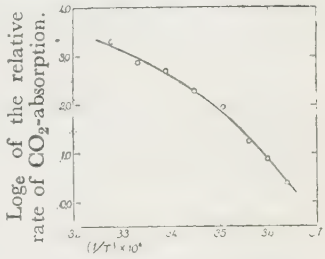



FIG. 6. Temperature-dependency of the dark reaction of bacterial photosynthesis. Hydrogen-door: butyrate.

## SUMMARY

1. It was found that a non-sulfur purple bacterium, *Rhodobacillus palustris* evolves molecular hydrogen when illuminated white weak light This process was shown to be the cause of the peculiar relationship found between the light intensity and the apparent rate of photosynthesis in purple bacteria.

2. The effect of various factors upon the process of photochemical $H_2$-evolution were investigated. It is suppressed by hydroxylamine, by treatment of bacterial cells with ultraviolet rays, by heating at 45°., and also retarded to some extent, but not completely, by ammonium ion and, presumably, by molecular nitrogen. Acceleration of the

process was brought about by provision of fumarate, succinate or pyruvate to bacterial suspension, while no such effect was observed with malate.

3. Using butyrate as a hydrogen-donor and eliminating the effect of $H_2$-production by placing palladium asbestos in the manometer vessel, the rate of photosynthetic $CO_2$-absorption was measured under various light intensities. The relationship thus found between the photosynthetic rate and the light intensity was essentially the same as that observed in green algae. A close analogy between the photosynthesis by purple bacteria and by green algae was also found in the temperature-dependency of the dark reaction, suggesting in both cases that the reaction involves at least two consecutive steps with widely different temperature coefficients. From the data obtained with the purple bacterium, the activation energies of these two reaction steps were computed. The values obtained (5–6 k cal. and 26–27 k cal.) were practically equal to these found earlier by Tamiya *et al.* for *Chlorella ellipsoidea.*

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# COLORIMETRIC DETERMINATION OF VITAMIN A. WITH GLYCEROL DICHLOROHYDRIN

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"Activated" Glycerol dichlorohydrin (GDH) was first introduced by Sobel *et al.* (*1*, *2*) for determining vitamin A. It is recommended as the color produced by the "activated" GDH is relatively stable, independent of saponification and the reagent is stable, non-corrosive and unaffected by moisture. Its defect is the relatively low sensitivity, being about 1/4 of the Carr-Price reaction and the relatively higher effect by carotenes than Carr-Price reaction. Feinstein (*3*) and Panketh (*4*) noticed the increased sensitivity by addition of hydrochloric acid and Antoniani (*5*) observed the increased coloration by addition of antimony trichloride to GDH. We examined the various conditions for color reaction of vitamin A with GDH and found that the so-called "activation" of GDH is nothing but the addition of antimony trichloride. The completely purified GDH gives no color reaction with vitamin A and the non-activated GDH gives the same color by addition of antimony trichloride. We found that the sensitivity of the color reaction is increased considerably by adding an appropriate amount of antimony trichloride and hydrochloric acid to GDH, the merits of GDH method being retained at the same time. The most satisfactory conditions which we reached are reported below.

## REAGENTS

(1) GDH containing 20–30 mg. antimony trichloride per ml. Commercial GDH is redistilled and the necessary amount of antimony trichloride is added. It is stored in a dark bottle in a dark place.

The used GDH reagent can be recovered by redistillation in total glass apparatus under reduced pressure (about 100 mm. Hg at 65–70°), the first small portion being discarded. The content of antimony trichloride in distillate is determined as follows: 1 ml. of the distillate is placed in a beaker. 20 ml. of K-Na-tartarate solution (containing 2 g.) are added and 2 g. of Na bicarbonate are mixed. The whole is titrated with $N/10$ iodine solution, starch being used as an indicator.

1 ml. of $N/10$ iodine corresponds to 11.4 mg. of $SbCl_3$. The appropriate amount of $SbCl_3$ is added to make 25 mg./ml. in final concentration.

(2) Chloroform. Analytically pure preparation.

(3) 5% KOH in ethyl alcohol. Ethyl alcohol must be free from peroxide. On adding rhodanide no color reaction should take place. If not, about 100 g. of $FeSO_4$ and small amount of KOH are added to 500 ml. of alcohol. After thorough mixing and standing overnight the supernatant is distilled.

4. 5% KOH in 60% methyl alcohol. Methyl alcohol must also be free from peroxide.

5. Benzene.

6. Concentrated hydrochloric acid.

7. Hydrogen gas.

8. Carbon dioxide gas.

## PROCEDURE

$a$ g. (usually 0.01) of liver oil is dissolved in $v$ ml. (usually 5) of chloroform. 1 ml. of this solution is poured into a small test-tube containing 2 ml. of GDH solution and 0.1 ml. of concentrated HCl. After thorough mixing it is placed in an 1 cm.-cuvette and the extinction E is measured by Pulfrich photometer (with filter $S_{55}$) within 2–10 minutes after addition of the reagents.

The vitamin content $x$ can be calculated from the following formula.

$$x=3{,}000\times\frac{Ev}{a}(\gamma/100\text{g.})=90\times\frac{Ev}{a}(\text{I.U./g.})$$

The color produced immediately after addition is blue, but it is rapidly changed to relatively stable purplish red color resembling permanganate, which is estimated photometrically.

## EXPERIMENTAL

*Studies of Sobel's Method*—1 ml. of vitamin A solution in chloroform containing 20 $\gamma$ is added to 4 ml. of the GDH (Wako-Junyaku, Osaka) at 25°, which had been activated according to Sobel (*1*). The mixture showed a faint blue color, which disappeared in several seconds, while the non-activated preparation gave no color. We estimated the $SbCl_3$ concentration of the activated preparation as described above and found it to contain $SbCl_3$.

We tested the non-activated GDH preparation after adding various

amount of $SbCl_3$ and found as shown in Table I that the maximal and stable E was obtained with GDH containing 0.5 g. of $SbCl_3$ per ml.

TABLE I

*Effect of $SbCl_3$ Concentration on GDH Method of Sobel*

| Concentration of $SbCl_3$ | Time showing stable reddish purple color after addition of reagents | *E* |
|---|---|---|
| *g./ml.* | *min.* | |
| 1 | 6—10 | 0.292 |
| 0.5 | 11—more than 15 | 0.292 |
| 0.25 | 5—13 | 0.121 |
| 0.125 | 9—more than 15 | 0.068 |
| 0.062 | 3—more than 15 | 0.026 |

Then we compared the activated and non-activated GDH after addition of $SbCl_3$ to 0.5 g. per ml. and found that no difference was detected between the two.

This shows clearly that the so-called activation is nothing other than adding $SbCl_3$ to GDH.

*Effect of Temperature*—To vitamin A solution (16 $\gamma$/ml. chloroform) are added at various temperatures (10, 20, and 30°, respectively) 2 ml. of GDH solution (26 mg. of $SbCl_3$ in 1 ml. of GDH) and 0.02 ml. of concentrated HCl. E values were estimated successively at regular intervals.

The E values have been found to be the same in all cases to reach maximum in 2 minutes, remaining constant as long as 10 minutes.

*Effect of $SbCl_3$ Concentration*—To 2 ml. of GDH containing various amount of $SbCl_3$ are added 1 ml. of chloroform solution containing 16 $\gamma$ of vitamin A. After further addition of 0.02 ml. of concentrated HCl the extinction was estimated successively. The results in Table II show that the maximal and stable E values are obtained at concentration of 0.01–0.05 g. of $SbCl_3$ per ml. of GDH.

*Effect of HCl Concentration*—0.02, 0.04, 0.08, 0.18, and 0.30 ml. of concentrated HCl are added respectively to 2 ml. of GDH solution (containing 26 mg. of $SbCl_3$ per ml.) and 1 ml. of chloroform solution (containing 10 $\gamma$ of vitamin A). After 3 minutes the extinctions were estimated. No difference was detected except in 0.30 ml., where the solution became turbid.

TABLE II

*Effect of $SbCl_3$ Concentration*

| Concentration of $SbCl_3$ | Time showing stable *E* value after addition of reagents | Initial *E* | Stable *E* |
|---|---|---|---|
| *g./ml.* | *min.* | | |
| 0 | 1—10 | 0.370 | 0.385 |
| 0.01 | 1—10 | 0.498 | 0.511 |
| 0.05 | 1—10 | 0.501 | 0.510 |
| 0.1 | 1 | 0.549 | 0.555 |
| 0.5 | unstable | 0.404 | 0.404 |

*Effect of Vitamin A Concentration*—1 ml. of various amounts of vitamin A was added to 2 ml. of GDH solution (containing 26 mg. $SbCl_3$ per ml.) and 0.02 ml. of concentrated HCl. 3 minutes after mixing the extinctions are estimated. As the results in Table III show, the extinctions are exactly proportional to the concentrations of vitamin A, the conversion factor being found to be 30.

TABLE III

*Effect of Vitamin A Concentration*

| Amount of vitamin A | E | $\gamma$/E |
|---|---|---|
| $\gamma$ | | |
| 10.0 | 0.335 | 29.8 |
| 5.0 | 0.167 | 29.9 |
| 2.5 | 0.083 | 30.2 |
| 1.25 | 0.042 | 29.8 |

*Absorption Curve of the Colored Solution*—Using 10 $\gamma$ of vitamin A the color was produced just in the same way as was described in the procedure. As is shown in Fig. 1, the maximal absorption was found to be at about 550 m$\mu$.

*Reaction of Carotene*—Carotene shows the same stable purplish red color in 2 minutes after adding the reagents and remains constant for 10 minutes. The E value is about 1/10 of vitamin A on the same weight basis.

*Effect of Saponification*—Unsaponifiable fractions of various liver oils were determined, and the values obtained were compared with those

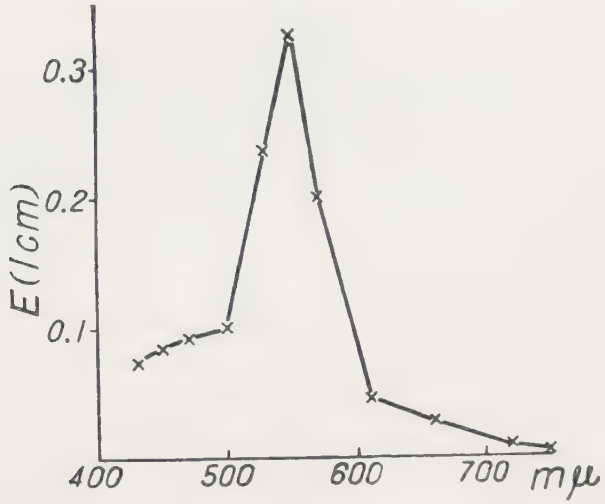


FIG. 1. Absorption curve of the colored solution.

determined without saponification. The results in Table IV show that saponification has no influence upon the vitamin values.

TABLE IV

*Effect of Saponification*

| Liver oils of | With saponification | Without saponification |
|---|---|---|
| *Balaenoptera physalus* | | |
| a) Molecular distillate | 16,600 | 16,400 |
| b) Original oil | 3,920 | 4,000 |
| *Reinhardtuis matsuurae* | 83,300 | 83,100 |
| *Scoliodon walbeehmi* | 4,450 | 4,400 |

*Comparison with Carr-Price Method*—The values determined as described in this paper were compared with those determined by Carr-Price method. Table V shows that both values agree practically completely.

*Comparison of Sensitivity*—Vitamin A content of liver oil was determined by various methods reported in the literature and the ratios $E/\gamma$ vitamin A were determined. The results in Table VI show that this method is more sensitive than any GDH methods hitherto reported, sensitivity being about 74% of Carr-Price method.

TABLE V

*Comparison with Carr-Price Method*

(Figures show vitamin content in I.U./g.)

| Method | GDH | Carr-Price |
|---|---|---|
| *Theragra chalcogramma* | 18,700 | 18,900 |
| *Squalus sacklevi* | 11,500 | 11,400 |
| *Reinhardtius matsuurae* | 2,560 | 2,590 |
| *Balaenoptera physalus* | 11,500 | 11,400 |
| *Katsuwonus vagans* | 5,570 | 5,510 |

TABLE VI

*Sensitivities of Various Methods*

| Method | $E/\gamma$ Vitamin A | Relative sensitivity |
|---|---|---|
| Sobel (*1*) | | |
| a) With purified GDH | 0 | 0 |
| b) With addition of 0.5 g. $SbCl_3$/ml. GDH | 0.0152 | 33 |
| Reinstein (*3*) | | |
| a) With purified GDH | 0.0050 | 11 |
| b) With addition of 26 mg. $SbCl_3$/ml. GDH | 0.0063 | 14 |
| Antoniani (*5*) | 0.0107 | 23 |
| Authors' method | 0.0334 | 74 |
| Carr-Price | 0.0455 | 100 |

SUMMARY

The "activation" of GDH of Sobel is nothing other than the effect of $SbCl_3$ which is contained in the reagents used. By the addition of an adequate amount of antimony trichloride and hydrochloric acid to GDH, an exact method of the vitamin A determination is established. This method has higher sensitivity than any other GDH methods hitherto reported, the merits of GDH methods being retained at the same time. The procedure and effects of various conditions were reported in detail.

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# FLUOROMETRIC DETERMINATION OF VITAMIN A

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(Received for publication, May 21, 1951)

That vitamin A has greenish yellow fluorescence in ultraviolet light has been reported by Kedvessy (*1*), Sobotka (*2*) and Shantz (*3*). According to the latter author, vitamin $A_1$ fluoresces brilliant yellow and $A_2$ brownish orange. Kedvessy (*1*) tried to apply the fluorescence to the determination of vitamin A by titrating with picric acid to disappearance of fluorescence. But Sobotka (*2*) observed the different intensity of fluorescence between free and esterified vitamin A and among vitamin A esters the intensity varies with different fatty acid components. He noticed also the lability of fluorescence of esterified vitamin A. Therefore this method seems to be applicable only to the pure solution of vitamin A alcohol or ester of the same fatty acid. We studied the fluorometric estimation of vitamin A of liver oils after converting the ester to free alcohol by saponification and a satisfactory method of determination was obtained, the sentivity of which is about 10 times as high as that of the ordinary colorimetric method.

## REAGENTS

(1) 5% KOH in ethyl alcohol.
(2) 5% KOH in 60% methyl alcohol.
(3) Benzene.
(4) Petroleum benzine.
(5) Acetone.
(6) 10% Acetone in petroleum benzine. All solvents must be free from fluorescence. It is easily accomplished by redistillation They must also be free from peroxide. If not, peroxide is reduced by ferrous sulfate preliminary to distillation.

(7) Activated alumina BL6. The same "activated alumina BL6", as is reported for separating vitamin D from vitamin A is used (Fujita and Aoyama (*4*)). After being washed with fluorescence-free methanol and petroleum benzine it is heated at 500° for 1 hour.

The "activated alumina KK1" of the Scientific Research Institute, Komagome-Kamifujimae-cho, Bunkyo-ku, Tokyo can also be used after being washed with water, spread on a dish, dried at room temperature for about 48 hours, and heated at 150° for one hour. (This can equally be used for separating vitamin D from A).

The particle size of the latter alumina being a little smaller than the former, the filtration is performed at a somewhat more reduced pressure.

(8) Standard vitamin A solution. A standard solution containing 100 $\gamma$ of vitamin A alcohol in 100 ml. is prepared, and is stored in $CO_2$ after being bubbled with $CO_2$.

## PROCEDURE

*Saponification and Extraction*—*a* g. of liver oil (usually 0.01) are dissolved in a centrifuge-tube with stopper in 5 ml. of 5% KOH in ethyl alcohol and the mixture is bubbled with hydrogen. After being heated at 75° for 30 minutes, it is cooled rapidly. 10 ml. of benzene and 10 ml. of water being added, mixed and centrifuged, the benzene layer is poured in another centrifuge-tube with stopper followed by washing with 20 ml. of 5% KOH in 60% methanol and then with water several times. The extract becomes usually completely transparent. If turbid, the washing is further continued. Each step is performed in hydrogen or $CO_2$. The benzene layer is transferred quantitatively to a test-tube and made up with benzene to *m* ml. It is further diluted *v* times with benzene to appropriate concentration (0.1–1.0 $\gamma$ vitamin A per ml.).

*Measurement*—Two similar test-tubes showing no fluorescence upon ultraviolet irradiation are prepared. 1 ml. of the test solution is poured in one tube, benzene being added to 5 ml.

In another tube containing 5 ml. of benzene standard vitamin A solution is added till the intensity of fluorescence matches with the other tube, the corresponding amount of benzene being added to the other tube.

*Calculation*—When the titrated volume of the standard vitamin solution is *t*, the content of vitamin A in oil *x* is calculated by the following formula.

$$x=\frac{tmv}{a}(\gamma/\text{g.})=\frac{3tmv}{a}(\text{I.U./g.})$$

Recovery of the added amount of vitamin A is practically complete, being 96–100%.

The fluorometric measurement can also be made by fluorometer, comparing with calibration curve of pure vitamin A solution.

The optimal vitamin amount of this method is 0.3–1.0 $\gamma$, while that of the GDH method reported by the author (5) is 3–15 $\gamma$ namely this is about 10 times more sensitive than the latter.

## EXPERIMENTAL

*Amount of Vitamin A and Intensity of Fluorescence*—The liver oil of Reinhardtius matsuurae (containing 110,000 I.U. of vitamin A per g.) is saponified and the vitamin A fraction is diluted to an appropriate concentration (containing 1 $\gamma$ vitamin A per ml.) and its various amount is made up with benzene to 5 ml. and is measured fluorometrically. The results in Table I show that the intensity of fluorescence is proportional to the amount of vitamin A.

TABLE I

*Amount of Vitamin A and the Intensity of Fluorescence*

| Vitamin A | Titrated volume |
|---|---|
| $\gamma$ | *ml.* |
| 0.25 | 0.25 |
| 0.50 | 0.50 |
| 0.75 | 0.749 |
| 1.0 | 1.004 |

*Fluorescence of Carotene, Cryptoxanthin and Vitamin $D_3$*—Carotene (from spinach), Cryptoxanthin (from juice of tangerine orange) and vitamin $D_3$ (from liver oil of " Maguro ") were isolated chromatographically and the intensity of fluorescence were measured.

As is shown in Table II. the intensity of carotenoids is about 12% and that of vitamin $D_3$ about 3% of vitamin A on weight basis.

TABLE II

*Fluorescence of Carotenoides and Vitamin $D_3$*

| Substance | Color of Fluorescence | Intensity in comparison with vitamin A. |
|---|---|---|
| Carotene | Yellowish green | 12% |
| Cryptoxanthin | Yellowish green with somewhat white tint | 12% |
| Vitamin $D_3$ | Greenish purple | 33% |

*Influence of Saponification*—The influence of saponification was studied with liver oil of Seriola quinqueradiata in case of fluorometric and GDH method.

The results in Table III show that the saponification has no influence upon GDH method, but in the fluorometric method vitamin A ester is about 20% more fluorescent than free alcohol on molar basis. Vitamin A acetate (American standard oil containing 10,000 I.U./g.) showed also about 20% more fluorescence after saponification.

TABLE III

*Influence of Saponification upon Vitamin A Values* (I.U./g.)

| Method | Without saponification (*a*) | With saponification (*b*) | *a*/*b* (%) |
|---|---|---|---|
| GDH | 12,800 | 12,600 | 102 |
| Fluorometric | 15,700 | 12,700 | 124 |

*Comparison of Vitamin A Values Estimated by GDH Method and by Fluorometric Method*—The unsaponificable fraction of various liver oils were estimated by GDH and fluorometric methods and the vitamin A values were compared. As shown in Table IV, the results obtained by both methods agree practically completely.

TABLE IV

*Comparison of GDH and Fluorometric Method*

(Figures given in the table show vitamin A content in I.U./g.)

| Liver oils of | GDH (*a*) | Fluorometric (*b*) | *a*/*b* (%) |
|---|---|---|---|
| *Seriola quinqueradiata* | 12,600 | 12,700 | 101 |
| *Squalus sackleyi* | 15,200 | 14,800 | 98 |
| *Scoliodon walbeehmi* | 4,500 | 4,530 | 101 |
| *Theragra chalcogramma* | 8,300 | 8,500 | 102 |
| *Katsuwonus vagans* | 2,520 | 2,700 | 107 |
| *Theragra chalcogramma* | 28,300 | 28,200 | 100 |
| *Reinhardtius matsuurae* | 99,000 | 100,800 | 102 |

SUMMARY

The yellowish green fluorescence of vitamin A can be effectively used for its estimation. The fluorometric method is about 10 times

more sensitive than the colorimetric. The intensity of fluorescence is proportional to the concentration. The intensity of carotene and cryptoxanthin is about 12% and vitamin $D_3$ about 3.3% of vitamin A on weight basis. The fluorescence is less intense after saponification. The fluorescence of free vitamin A alcohol is about 20% less intensive than A ester on molar basis. The vitamin A values, determined after saponification by GDH and fluorometric method agree practically completely.

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# STUDIES ON LIPASE. II. ON THE HISTIDINE ACTIVATION OF PANCREAS LIPASE

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In the previous paper (*1*) the author has reported the finding that the pancreas lipase, extracted from the acetone powder of pig pancreas, is notably stimulated by the addition of several amino acids and some organic acids, such as succinic and citric acid, and that histidine has an actually remarkable activating effect on the pancreas lipase, showing more than 100 per cent activation. This report is concerning with further investigations on histidine activation of the pancreas lipase.

Regarding the lipase components, Rosenheim (*2*) (1910) reported a separation of the pancreas lipase into co-enzyme and prolipase, which had no more hydrolysing activity. According to Woodhause (*3*) (1932), prolipase was reactivated not only by extract of boiled pancreas, but by blood serum of various animals, and also by lead ion. R. Itoh (*4*) (1936) found in ricinus seeds a substance, which controlled the synthetic and hydrolytic action of the ricinus lipase, terming it " co-lipase "

It must be here also cited that Kobayashi (*5*) (1949) described L-histidine as a co-phospho-monoesterase.

In these experiments the author has succeeded by dialysis to separate the pancreas lipase into two components, namely an inactive component, remained in the dialysed solution, and a component, which is dialysable, found in the concentrated dialysate, and able to reactivate the former.

On $HgSO_4$ precipitation from this concentrated dialysate, it is demonstrated that this fraction shows a reactivating effect on the dialysed inactive lipase solution, and a crystalline L-histidine dipicrate is isolated from this fraction.

On the other hand, the inactive component of dialysed solution, perhaps apo-lipase, is remarkabley reactivated by the addition of L-histidine.

These results suggest a coenzymatic role of L-histidine in the pancreas lipase.

## EXPERIMENTALS

*L-Histidine Effect on Hydrolysis of Various Esters by Pancreas Lipase*—Enzyme: 0.7 g. acetone powder of pig's pancreas were extracted with 200 ml. of 70% glycerol water, adjusted to pH 8.6 with dilute ammonia, and incubated at 37° for 3 hours and centrifuged.

This supernatant fluid was used as a lipase solution in the experiments.

Test solution: It contains 2 ml. of 0.2 *M* $NH_4Cl$-$NH_4OH$ buffer (pH 8.6), 2 ml. of lipase solution, 1 ml. of 0.1 *M* histidine solution and 0.17 g. of triacetin or 0.23 g. of tributyrin or 0.11 g. of butyl-butyrate.

Control solution: It consists of all other components except histidine.

Blanks without enzyme or without substrate were examined.

Each solution was incubated under toluene at 37° for 3 and 24 hours, and liberated acid in 1 ml. digest was titrated with 0.1 *N* NaOH by a microburette in 1 ml. digestate. The values corrected for blanks are given as the acidity increase in the tables, and the percentage of the values of the main tests to the value of the controls is given as the degree of hydrolysis in the parentheses in the tables.

TABLE I

Acidity increase in 1 ml. of digest at pH 8.6 (ml., 0.1 *N* NaOH)

| Substrates / Time (hrs.) / Addition | Triacetin | | Tributyrin | | Butyl-butyrate | |
|---|---|---|---|---|---|---|
| | 3 | 24 | 3 | 24 | 3 | 24 |
| Control (No addition) | Degree 0.320 (100) | Degree 0.640 (100) | Degree 0.610 (100) | Degree 0.885 (100) | Degree 0.175 (100) | Degree 0.320 (100) |
| Histidine (0.02 *M* final concentration) | 0.500 (156) | 0.970 (147) | 1.040 (170) | 1.300 (147) | 0.175 (100) | 0.320 (100) |

*Effect of L-Histidine and DL-histidine* on Olive Oil Hydrolysis by Pancreas Lipase*—Test solution: It consists of 3 ml. of 0.2 *M* $NH_4Cl$-$NH_4OH$ buffer of pH 8.6+3 ml. of L- or DL-histidine solution of different concentrations+2 ml. of 1% $CaCl_2$ solution+2 ml. of lipase solution+

* DL-Histidine was prepared according to E. Abderhalden and A. Weil (*6*) (1912).

5 ml. of olive oil in the polyvinyl alcohol solution.* The final concentration of olive oil was about $M/50$ assuming that olive oil is triolein. It was adjusted to pH 8.6 and incubated under toluene at 37° for 5 hours.

Blanks were examined as in the previous tests. The acidity increase, corrected for blanks, is recorded in Table II.

TABLE II

Acidity increase in 5 ml. of digest (olive oil) at pH 8.6 in 5 hours (ml., 0.1 $N$ NaOH)

| Additions \ Final concentration | 0 | $M/200$ | $M/100$ | $M/50$ | $M/25$ |
|---|---|---|---|---|---|
| L-Histidine | 0.210 | 0.430 | 0.720 | 0.960 | 1.020 |
| DL-Histidine | 0.210 | 0.410 | 0.730 | 0.950 | 1.010 |

*Optimal Concentration of L-histidine for Hydrolysis of Triacetin and Olive Oil by Pancreas Lipase at pH* 8.6—Enzyme for triacetin test: 0.2 g. pancreas powder were transfered in 100 ml. of 70% glycerol-water and kept under stirring at 37° for three hours.

The extract was centrifuged and the supernatant fluid was used in the experiments.

Test solutions: 2 ml. of $M/8$ triacetin solution**+2 ml. of lipase extract +2 ml. of L-histidine solution of various concentrations +4 ml. of 0.2 $M$ $NH_4Cl$-$NH_4OH$ buffer of pH 8.6 (final concentration of triacetin was 1/40 $M$).

Controls without histidine solution but with 2 ml. water.

Enzyme for olive oil test: 0.5 g. pancreas powder were extracted with 100 ml. of 70% glycerol-water and the centrifuged fluid was used.

Test solutions: 5ml. of olive oil emulsoid*** +3ml. of 0.2 $M$ $NH_4Cl$-$NH_4OH$ buffer (pH 8.6)+2 ml. of 1% $CaCl_2$ solution +3 ml. of L-histi-

* The procedure concerning the polyvinyl alcohol solution of olive oil was described in the previous paper (Report I) (*1*).

** 2.276 g. of redistilled triacetin was under shaking disolved in 100 ml. of water ($M/8$).

*** 5.6 g. of olive oil was shaken with 160 ml. of 1% polyvinyl alcohol solution for one hour, in order to make an oil emulsoid of about $M/17$, calculated as triolein.

dine solution of various concentrations +2 ml. of lipase solution. (final concentration of olive oil was about $M/51$).

Controls without histidine but with 3 ml. of water.

Blanks without substrate or without enzyme. The test solutions were digested at 37°. The acidity increase in the digest and the degree of hydrolysis are given in the Table III.

TABLE III

| Acidity increase in 2 or 5 ml. of digest (ml., 0.1 $N$ NaOH) | | | | |
|---|---|---|---|---|
| Substrates / Time (hours) / L-Histidine in final $M$ concentration | 2 ml. triacetin digestate | | 5 ml. olive oil digestate | |
| | 2 | | 24 | |
| Control (No addition) | 0.176 | Degree (100) | 0.255 | Degree (100) |
| 0.0025 $M$ | 0.217 | (123) | 0.617 | (242) |
| 0.005 $M$ | 0.320 | (182) | 0.805 | (315) |
| 0.01 $M$ | 0.435 | (247) | 1.055 | (413) |
| 0.02 $M$ | 0.456 | (259) | 1.160 | (457) |
| 0.025 $M$ | 0.579 | (329) | 1.160 | (457) |
| 0.05 $M$ | 0.486 | (276) | 1.170 | (458) |
| 0.1 $M$ | 0.380 | (216) | —— | —— |

*Effect of* L-*Histidine and Histamine on Triacetin Hydrolysis by Pancreas Lipase*—Test solutions: 0.17 g. of triacetin in 3 ml. $M/30$ tyrosine or tyramine buffer solution of pH 8.6+2 ml. of lipase solution or 0.17 g. of triacetin in 2 ml. buffer +1 ml. of 0.1 $M$ L-histidine or histamine solution +2 ml. of lipase solution; incubated under toluene at 37° for 1 and 6 hours.

Controls without additions and blanks without enzyme or without substrate are carried out in the experiments.

The acidity increase in 2 ml. of digest as well as the degree of the hydrolysis are recorded in Table IV.

*Separation of Pancreas Lipase into Apo- and Co-Ferment by Dialysis*—Dialysis procedure: 1 g. of acetone powder of pig's pancreas, prepared in an already described manner, was suspended in a mixture of 2 ml. of 90% glycerol and 13 ml. of water, stirred at 37° for 4 hours and then

TABLE IV

| Acidity increase in 2 ml. of digest (triacetin) at pH 8.6 (ml., 0.1 *N* NaOH) | | | | |
|---|---|---|---|---|
| Additions (0.02 *M* in final concentration) / Time (hours) | 1 | | 6 | |
| Control (No addition) | 0.510 | Degree (100) | 0.920 | Degree (100) |
| L-Histidine | 0.870 | (170) | 1.770 | (192) |
| Histamine | 0.660 | (129) | 1.260 | (137) |
| L-Tyrosine | 6.670 | (133) | 1.260 | (137) |
| Tyramine | 0.510 | (100) | 0.920 | (100) |

centrifuged. The supernatant fluid* was dialysed as the lipase solution in a collodium sack against 500 ml. of water for 2 hours.**

This dialysed lipase solution (about 30 ml.) was filled up to 50 ml. with 90% glycerol and examined for on its activity, as an apo-enzyme fraction.

The dialysate was concentrated to about 40 ml. at 40°.

One ml. of this residual fluid was added to the dialysed inactive lipase solution, to be examined for its activating influence (A).

The greater part of the dialysate was acidified with 5% sulfuric acid, and precipitation was carried out with 10% $HgSO_4$ solution. The separated precipitation was suspended in water and aerated with $H_2S$ gas. The filtrate, freed from mercuric sulfide, was evaporated to about 10 ml. 1 ml. of this solution was used in the experiments of reactivation (B).

The residual solution, adjusted to pH 6, was mixed with picric acid and on standing some yellow crystals were precipitated. After recrystalisation it melted at 85° and showed no depression with L-histidine dipicrate (m.p. 85°).

Test solution: 0.17 g. triacetin in 2 ml. of 0.2 *M* $NH_4Cl$-$NH_4OH$ buffer (pH 8.6) + 2 ml. of dialysed lipase solution, mixed with 1 ml. dialysate concentrated (A) or 1 ml. of $HgSO_4$ precipitation fraction (B) described above.

* Each fluid of this solution gave a strong Pauli's diazo reaction.

** A prolonged dialysis seems to totally damage apo-lipase.

Control solution: It contains 2 ml. of original lipase solution, which was not dialysed but filled up to 50 ml. with glycerol, and 1 ml. of water without dialysate.

Blanks without enzyme or without substrate. The acidity increase and the degree of hydrolysis in 2 ml. digest are recorded in Table V.

TABLE V

| Dialysed lipase and its dialysate. | Acidity increase in 2 ml. of digest (triacetin) at pH 8.6 in 24 hours. (ml., 0.1 $N$ NaOH) | |
|---|---|---|
| Control: Original lipase solution not dialysed | 0.510 | (Degree of hydrolysis) (100.0) |
| Dialysed lipase+water | 0.050 | (9.8) |
| (A) Dialysed lipase+dialysate | 0.450 | (88.2) |
| (B) Dialysed lipase+$HgSO_4$ precipitation fraction from dialysate | 0.360 | (70.6) |

*Reactivation of the Dialysed Inactive Pancreas Lipase by Addition of* L-*Histidine of Vairous Concentrations*—Dialysed lipase solution: The same solution, as described in Table V, was used.

Test solution: 0.17 g. triacetin in 2 ml. of 0.2 $M$ $NH_4Cl$-$NH_4OH$ buffer (pH 8.6)+2 ml. of dialysed lipase solution+1 ml. of L-histidine solution of various concentrations in a range from 0.005 to 0.1 $M$.

Control: 2 ml. of original lipase solution, examined in Table V, were used in this experiment.

Blanks without enzyme or without substrate. The acidity increase and the degree of hydrolysis are given in Table VI.

*Effect of* L-*Histidine on Triacetin Hydrolysis by a Crude Pancreas Maceration*—Pancreas maceration: 10g. of fresh minced pancreas of a pig were ground vigorously with 100 ml. of 70% glycerol-water and kept under toluene in an ice box overnight. The crude maceration was filtered through gauze and the filtrate was mixed with the same volume of 70% glycerol-water and centrifuged.

The supernatant fluid was used as a lipase source.

Test solution: 0.17g. of triacetin+2 ml. of $NH_4Cl$-$NH_4OH$ buffer (pH 8.6)+2 ml. of pancreas maceration+1 ml. of 0.1 $M$ L-histidine solution.

TABLE VI

| Dialysed lipase and final concentration of L-histidine added (*M*) | Acidity increase in 2 ml. of digest (triacetin) at pH 8.6 in 24 hours. (ml., 0.1 *N* NaOH) | |
|---|---|---|
| Control: | | (Degree of hydrolysis) |
| Original lipase solution not dialysed | 0.510 | (100.0) |
| Dialysed lipase+water | 0.050 | (9.8) |
| Dialysed lipase+L-histidine (0.005 *M*) | 0.090 | (17.0) |
| Dialysed lipase+L-histidine (0.01 *M*) | 0.160 | (31.4) |
| Dialysed lipase+L-histidine (0.02 *M*) | 0.280 | (55.7) |
| Dialysed lipase+L-histidine (0.05 *M*) | 0.410 | (80.4) |
| Dialysed lipase+L-histidine (0.1 *M*) | 0.480 | (94.1) |

Controls without L-histidine, but with 1 ml. of water.

Blanks without enzyme or without triacetin. The test solution was incubated under toluene at 37° for one and 24 hours. The acidity increase, corrected for blanks and the hydrolysis degree are listed in Table VII.

TABLE VII

| Additions \ Time (hours) | Acidity increae in 1 ml. of digest at pH 8.6 (ml., 0.1 *N* NaOH) | | | |
|---|---|---|---|---|
| | 1 | | 24 | |
| Control (No addition) | 0.621 | Degree (100) | 1.230 | Degree (100) |
| L-Histidine (0.02 *M* final concentratian) | 0.850 | (137) | 1.710 | (139) |

*Reactivation of the Dialysed Pancreas Maceration by Addition of Dialysate or L-Histidine*—Dialysis of pancreas maceration: 15ml. of glycerol maceration of pig's pancreas, used in experiment VII (10 g. ground pancreas in 100 ml. of 70% glycerol) were dialysed in a collodium sack against 500ml. of distilled water at room temperature for 2 hours and again against 500 ml. of renewed water for 4 more hours (six hours in all). The dialysed pancreas maceration, which had been increased to about 40 ml., was filled up to 50 ml. with 90% glycerol water and used in the experiments.

The dialysate (about 1,000 ml.) was evaporated to about 10 ml. at 40° under reduced pressure and tested for its reactivating influence.

Test solution: 2 ml. of 0.25*M* triacetin solution+2 ml. of $NH_4Cl$-$NH_4OH$ buffer (pH 8.6)+4 ml. dialysed pancreas maceration+2 ml. of L-histidine solution of various concentrations or 2 ml. of dialysate.

Controls: Here was used the original, not dialysed pancreas maceration, 15ml. of which were filled up to 50ml. with 25ml. of water and 10 ml. of glycerol water.

Blanks were tested without pancreas maceration or without dialysed maceration or without triacetin.

These solutions were digested under the same conditions as in the previous experiments.

TABLE VIII

| Crude pancreas maceration dialysed for 6 hours, and mol final concentration of L-histidine added | Acidity increase in 2 ml. of digest (triacetin) at pH 8.6 in 2 or 24 hours (ml., 0.1 *N* NaOH) | | | |
|---|---|---|---|---|
| | 2 hours | | 24 hours | |
| | Acidity increase | Degree | Acidity increase | Degree |
| Control: Original pancreas maceration not dialysed | 0.810 | (100.0) | 1.650 | (100.0) |
| Dialysed crude pancreas maceration + Water | 0.300 | (37.0) | 0.650 | (39.4) |
| Dialysed crude pancreas maceration + Dialysate | 0.440 | (54.3) | 0.950 | (57.6) |
| Dialysed crude pancreas maceration + L-Histidine (0.05 *M*) | 0.450 | (54.6) | 0.970 | (58.8) |
| Dialysed crude pancreas maceration + L-Histidine (0.025 *M*) | 0.430 | (53.1) | 0.910 | (55.1) |
| Dialysed crude pancreas maceration + L-Histidine (0.01 *M*) | 0.400 | (49.4) | 0.820 | (49.7) |

## RESULTS AND DISCUSSION

It is generally known that the pancreas lipase is preferably adapted to hydrolysis of natural fats, or higher glycerides, but in a lower degree to that of synthetic mono alcohol esters.

The stimulating action of histidine is also ascertained in the hydrolysis of a glyceride of a lower organic acid such as triacetin or tributyrin, while it does not occur in the hydrolysis of butylbutyrate (Table I).

It is reported by Willstätter *et al.* (7) (1924), that L-leucyl diglycine shows much stronger acceleration on the lipase action than its optical antipode, D-peptide. Observations, however, indicate that there is no difference between L-histidine and DL-histidine referring to its activating influence on the lipase action on olive oil (Table II).

From the results of Table III it is clear that there exists a certain relationship between the concentration of the substrate and that of histidine; the maximal hydrolysis of triacetin of 0.025 *M* solution, and that of olive oil of about 0.02 *M* were found at the concentration of L-histidine of 0.025, and 0.02 *M*, respectively.

As Table IV shows, histamine seems to be inferior to histidine in its stimulating effect on the lipase hydrolysis of triacetin. Tyrosine possesses a moderate stimulating effect on lipase, but tyramine never does.

These facts demonstrate that the carbonyl of these amino acids is responsible to a certain extent for the activation of the lipase.

It is worth while to study whether histidine coexists with lipase in pancreas tissue or not. There was found a strong Pauli's diazo reaction in the water extract of the acetone powder, used as a lipase source. In reality the author has succeeded to isolate histidine monochlorhydrate from water extract of the acetone powder, namely about 0.1 g. histidine monochlorhydrate from 1 g. acetone powder.

On the other hand histidine dipicrate was also isolated from the acetone solution, which was used for drying treatment of pig's pancreas (about 2 g. from an acetone mixture used for about 250 g. pancreas).

These observations suggest that histidine should have some significance for the lipase, especially in the role of its activation.

From the results of dialysis of the enzyme it is ascertained, that the dialysed lipase solution loses almost its total activity, the degree of hydrolysis being less than 10% of the original hydrolysis by not-dialysed lipase. The lipase was reactivated about to its 90% degree of hydrolysis by the addition of the dialysate, concentrated to an small amount. It was also reactivated by the fraction of $HgSO_4$ precipitation from the dialysate, from which histidine dipicrate was isolated.

It is now evident that the pancreas lipase is separated by dialysis into two portions, namely an inactive dialysed lipase and a dialysate, which acts reactivating the dialysed lipase, and from which histidine dipicrate was isolated.

It is important to investigate the relationship between histidine concentration and its reactivation degree. The dialysed lipase, which

has a negligible activity, appeared to be influenced even by a very low concentration of histidine such as 0.005 *M;* and more than 90% reactivation was observed when histidine was added in 0.1 *M* final concentration. These results lead to a conclusion that histidine plays important role in the way of the activation of the pancreas lipase and also of reactivation for the dialysed inactive lipase component, presumably as a coenzymatic factor.

It is obvious from Table VII that the crude maceration of pig's pancreas or the glycerol-water extract of a fresh pancreas undergoes a weaker stimulation by the histidine addition than the extract of the pancreas powder. This might be due to the presence of enough histidine in the crude extract.

When the crude pancreas extract was dialysed in a collodium sack for six hours, it lost about 60% activity, but it was only weakly and in about the same degree reactivated by the addition of the dialysate or of histidine (Table VIII). On dialysis, however, the extract of the acetone powder loses almost all its activity but it is reactivated to about 90% by the addition of the dialysate or histidine (Table V, VI).

These behaviors of a crude maceration suggest that it contains an unstable apo-lipase, especially for dialysis in spite of its higher histidine content. In this connection reference must be made to the report of H. Kraut *et al.* (*8*) (1934) that the lipase has an unstable agon, which is converted into an anagon, that does not unite with the pheron, to make a complete symplex. On the contrary, Euler suggests an unstable apoferment namely pheron.

The above observations that the crude pancreas maceration easily undergoes inactivation, which cannot be effected by the dialysate or histidine, might indicate that the trypsin action of the crude maceration might be responsible for the inactivation of the lipase pheron, destroying the nature as carrier of protein.

## SUMMARY

1. The acetone powder of pig pancreas was extracted with 70% glycerol water, and the supernatant fluid was used as the lipase solution in the experiments. L-Histidine solution stimulates the lipase action at pH 8.6 to about 56% for triacetin and 70% for tributyrin hydrolysis, but not for butyl-butyrate.

2. No difference is found between L- and DL-histidine in respect to its activation of the pancreas lipase in the range of from 0.005 to

0.04 *M* final concentration of hisitdine, showing parallelism between the activation and the histidine concentration. There exists a relationship between the optimal concentrations of histidine and the maximal hydrolysis of triacetin and olive oil.

3. Regarding the activating effect of amino acids and their amine derivatives, effect of histamine (about 30%) is lower than histidine (70%), and that of tyramine is not ascertained, while tyrosine has yet some effect (about 30%).

4. The activity of the lipase solution (extract from pig pancreas powder giving a strong Pauli's diazo reaction) becomes after dialysis less than 10% of the original degree of the hydrolysis and it is reactivated by the addition of the concentrated dialysate, and also by the addition of L-Histidine solution. L-Histidine picrate was isolated from the concentrated dialysate.

5. With the crude maceration of the fresh pig pancreas a weak imulating effect of histidine was found.

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# FURTHER STUDIES ON PYROCATECASE*

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In a previously reported experiment in this laboratory (*1*, *2*), a cell-free enzyme, pyrocatecase, was isolated and fairly purified from the acetone-dried cells of a strain of *Pseudomonas sp.*, which was adapted to anthranilic acid. This enzyme catalyzes the oxidative rupture of benzene ring of catechol, yielding *cis-cis* muconic acid as the end product. Stanier *et al.* (*3*) showed that the acetone powder of a strain of *Pseudomonas*, grown on benzoic acid, had the enzymatic activity on catechol oxidation, and they isolated and identified β-ketoadipic acid as the end product of this reaction. The enzyme preparation used by them was the suspension of the dried cells, while in our experiments a fairly purified enzyme was used. The oxygen uptake was the same in both cases (one mole of oxygen per mole of the substrate), while the end products were different.

It is reasonable to consider that catechol may be converted to β-ketoadipic acid via *cis-cis* muconic acid. For these reasons, we re-examined our earlier experiments and obtained some interesting results concerning the nature of pyrocatecase.

## EXPERIMENTAL

*Material*—As reported in the previous paper (*1*), we used as the source of enzyme the acetone dried cells of *Pseudomonas* grown on media containing 0.2 per cent anthranylic acid for 12 hours.

*Preparation of the enzyme*—The purification of the enzyme was somewhat modified and simplified as compared with that reported previously. One gram of the acetone-dried cells was suspended in 50 ml. of *M*/15 phosphate buffer (pH 8.0), stored in an ice chest for 24 hours, and centri-

* The compendium of this article was announced ate the Annual Meeting of the Society of Japanese Biochemists in April 1951 at Tokyo University.

fuged at 17000 r.p.m. for 20 minutes, the supernatant being used as the enzyme A (adjusted at pH 7.2). The enzyme A solution was then fractionated with 0.3 and 0.6 saturated ammonium sulfate and the precipitate formed was dissolved in 5 ml. of $M/5$ glycylglycine buffer (pH 7.4). This solution was used as the enzyme B. This enzyme solution was further dialyzed against distilled water for over 20 hours in a collodion bag. This solution was used as the enzyme C.

*Measurement of the Enzymatic Reaction*—Oxidation of the catechol was measured by the usual manometric technique in Warburg vessel at 38°. $\beta$-Ketoadipic acid was determined by the aniline citric acid method (*3*, *4*).

*The Nature of Pyrocatecase*—Ferrous ion as the components of this enzyme:

Both the enzymes A and B oxidize catechol consuming oxygen always in theoretical amount. But the enzyme C obtained does not oxidize catechol completely. This fact does not accord with our early report. (*1*)

It is, however, very intersting that, as shown in Fig. 1 the enzyme C restores its full activity in the presence of ferrous sulfate (end concentration $10^{-3}M$). Ferric ion and other metallic ions such as $Zn^{\cdot\cdot}$, $Mn^{\cdot\cdot}$, $Co^{\cdot\cdot}$, and $Mg^{\cdot\cdot}$ were found to be incapable of replacing the ferrous ion in causing to the full restoration of the enzyme activity. These are shown in Figs. 1 and 2.

From the above experiments, it is realized that one of the components of pyrocatecase is ferrous ion. In respect to the necessity of ferrous ion for the enzyme action, the enzyme pyrocatecase is analoguous to the homogentisicase reported earlier in this laboratory (*5*). In the case of latter enzyme, it was very remarkable that $\alpha\alpha'$-dipyridyl inhibited the enzyme action immediately after the addition of this reagent. But in the case of pyrocatecase, $\alpha\alpha'$-dipyridyl exerted no inhibiting action at all in all the stages of enzyme purification, *i. e.* in the enzymes A, B, and C. If enzyme solution C was mixed previously with $\alpha\alpha'$-dipyridyl (end concentration, $3\times10^{-3}M$) and then supplied with both ferrous sulfate and substrate at the same time, total inhibition of enzyme reaction was observed.

It may be supposed that in this case the enzyme combines with ferrous ion forming a more hardly dissociable complex than in the case of homogentisicase, but a slowly dissociable one on long lasting dialysis. These are shown in Fig. 3 and Table I.

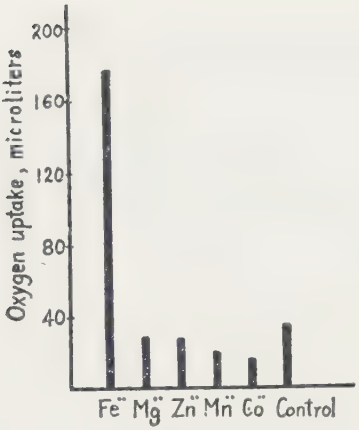


Fig. 1

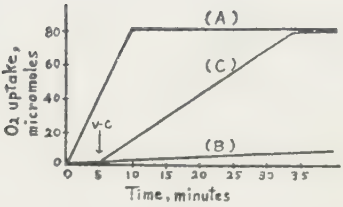


Fig. 2

Fig. 1. The effect of metallic ions on the catechol oxidation by enzyme C solution.

Each ion was used as sulfate.
Main compartment:

| | |
|---|---|
| Enzyme C solution | 1.0 ml. |
| *M*/15 phosphate buffer | 1.3 ml. |
| *M*/100 metalic sulfate | 0.3 ml. |

(control: no addition of any above metallic ions.)
Side arm:

| | |
|---|---|
| *M*/50 catechol | 0.4 ml. |

The columns show the amount of oxygen uptake in 30 minutes.

Fig. 2. The effect of iron ions on the catechol exidation by enzyme C.
Main compartment:

| | |
|---|---|
| Enzyme C solution | 1.0 ml. |
| *M*/15 phosphate buffer | 0.6 ml. |
| *M*/100 ferrous sulfate | 0.2 ml. (Curve A) |
| ,, ferric sulfate | 0.2 ml. (Curves B and C) |

Side arm I

| | |
|---|---|
| *M*/50 catechol | 0.2 ml. |

Side arm II

| | |
|---|---|
| *M*/50 ascorbic acid | 0.2 ml. (Curve C) |

(This reagent was tipped in 5 minutes after the start.)

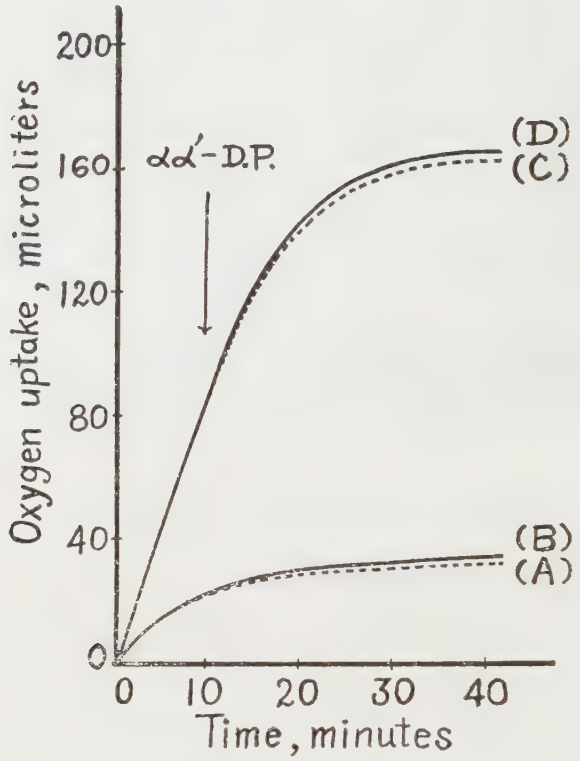


Fig. 3. The effect of αα'-dipyridyl (D.P.) on the pyrocatecase activity. Enzyme C solution, 1.0 ml.; *M*/15 phosphate buffer (pH 7.4), 1.0 ml.; *M*/100 $FeSO_4$, 0.3 ml.; *M*/30 D. P., 0.3 ml.; *M*/50 catechol, 0.4 ml. Curve A: Enzyme, buffer, and substrate were mixed without ferrous sulfate. Curve B: Enzyme and buffer were previously mixed with D. P., and then substrate and ferrous sulfate were tipped in. Curve C: D. P. was added 10 minutes after the substrate was tipped. Curve D. (control). Enzyme, buffer, and ferrous sulfate were mixed previously, then substrate was tipped in at the start.

TABLE I

*The Effect of Ferrous Ion on the Recovery of Enzyme Activity after Prolonged Dialysis*

The volume of the enzyme solution was increased 4 to 4.8 times of the initial volume during prolonged dialysis, so the volumes of the enzyme solution used were corrected so as to have same amounts of enzyme protein to each other. With and without 0.3 ml. of *M*/100 ferrous sulfate. *M*/50 Catechol, 0.4 ml. (Side arm) Total volume was made to be 3.0 ml. with *M*/25 phosphate buffer (pH 7.2).

| Time of dialysis | Volume of enzyme used | $FeSO_4$ added | $O_2$ uptake for 10 minutes |
|---|---|---|---|
| *hours* | *ml.* | *ml.* | *μl.* |
| 0 | 0.25 | 0.3 | 115 |
| | 0.25 | 0 | 83 |
| 15 | 1.0 | 0.3 | 114 |
| | 1.0 | 0 | 41 |
| 30 | 1.25 | 0.3 | 112 |
| | 1.25 | 0 | 7 |

*End products of the Enzyme Reaction on the Catechol Oxidation*—Isolation of β-ketoadipic acid: β-ketoadipic acid was isolated according to the procedure of Stanier (*3*). We could thus obtain about 50 mg. of pure acid from 250 mg. of catechol. The properties of this crystal are identical with those of β-ketoadipic acid reported by Stanier. But when we used the fractionated and dialyzed enzyme, the end product was not identical with β-ketoadipic acid, but with *cis-cis* muconic acid.

Isolation of the end product of the purified enzyme reaction: The end product was isolated according to the method in our previous report (*1*) with slight modification. In this experiment the enzyme C was used. The composition of the reaction mixture was as follows:

| | |
|---|---|
| Purified enzyme solution (enzyme C) | 60 ml. |
| Catechol | 250 mg. |
| *M*/15 phosphate buffer (pH 7.4) | 60 ml. |
| *M*/50 $FeSO_4 \cdot 7H_2O$ | 8 ml. |

The flask was shaken in a water bath at 38°. To avoid shifting of pH of the liquid towards acid side, diluted NaOH solution was pipetted in at intervals of 10 minutes. After the phenolic reaction in the small sample of fluid had decreased to a minimum value, the flask was removed from the water bath. The reaction mixture was shaken with chloroform several times, then concentrated $H_2SO_4$ was added to bring

the pH of the fluid to 2.8, and the flask was cooled immediately with ice. The reaction mixture was extracted with ether and the etheral extract was treated as reported earlier. Fine needles appeared. Melting point: 174°.

This substance was hardly soluble in water, easily in alkali solution, soluble in ether and in alcohol. Phenolic reaction and Rothera's reaction were negative. No $CO_2$ was evolved by catalytic decarboxylation with aniline citrate. It may be supposed that it is not β-ketoadipic acid as in Stanier's report (*3*), but is *cis-cis* muconic acid as already reported.

*Biochemical Behavior of the End Product Obtained and β-Ketoadipic Acid*—β-Ketoadipic acid was not oxidized further by living cells adapted to catechol. Also the preparation (*cis-cis* muconic acid) obtained above was not further oxidized by the same living cells. (With living cell suspension seven or more atoms of oxygen per mole of substrate were measurable for the complete oxidation of catechol).

But it was remarkable that our preparation (*cis-cis* muconic acid) was converted to β-ketoadipic acid without consumption of molecular oxygen by the action of the acetone-dried preparation of adapted cells. This transformation could not be brought about by the acetone-dried preparation of non adaptive cells. This is shown in Table II.

It is, however, not known at present why these substances, such as β-ketoadipic acid and *cis-cis* muconic acid, are not oxidized by living cells adapted to catechol. One of the tentative suppositions may be the impermeability of the cell membranes.

## DISCUSSION

It has been reported by Stanier as well as by us that catechol is an intermediate in the oxidation of benzoic acid or tryptophane and anthranilic acid, and in the course of the reaction benzene ring is ruptured, with the formation of dicarboxylic acid.

As the intermediate, Stanier obtained the same compound as synthesized β-ketoadipic acid with a crude enzyme, while we obtained *cis-cis* muconic acid with a purified enzyme.

It is natural to consider that *cis-cis* muconic acid lies on the metabolic pathway from catechol to β-ketoadipic acid. We can separate these enzyme reaction into two parts, one is the course from catechol to *cis-cis*-muconic acid, another is that from *cis-cis* muconic acid to β-ketoadipic acid.

TABLE II

*The Formation of β-Ketoadipic Acid from cis-cis Muconic Aacid by Acetone Dried Preparation of Catechol Adapted and Non Adapted Cells.*

A. For substrate oxidation—

Main compartment: 0.1 ml. of acetone-dried cell suspension (10 mg./1 ml. $H_2O$) or enzyme A solution; 1.0 ml. of *M*/15 phosphate buffer (pH 7.4).

Center cup: 0.2 ml. of 10 per cent KOH.

Side arm I: 0.2 ml. of *M*/50 catechol or isolated *cis-cis* muconic acid.

These were incubated for 30 minutes at 38° for the oxidation of the substrates, and then the formation of β-ketoadipic acid was determined as follows.

B. For catalytic decarboxylation by anilin citrate method (*4*)—

Main compartment: 0.3 ml. of 50 per cent citric acid was added.

Side arm II: 0.4 ml. of anilin citrate mixture.

(β-Ketoadipic acid produced was determined by $CO_2$ released.)

| Enzyme | Substrate | $O_2$ uptake | $CO_2$ released |
|---|---|---|---|
| | | μl. | μl. |
| Acetone-dried prepn. (adapted) | (1) catechol | 86.4 | 81.0 |
| | (2) *cis-cis* muconic acid | 1.8 | 83.0 |
| Cell-free prepn. (adapted) | (1) catechol | 88.0 | 85.0 |
| | (2) *cis-cis* muconic acid | 0 | 88.5 |
| Acetone dried prepn. (non adapted) | (1) catechol | 7.0 | 0 |
| | (2) *cis-cis* muconic acid | 2.0 | 0 |

Theoretical value for the complete oxidation of catechol or the complete decarboxylation of β-ketoadipic acid is 90 μl.

In the process of purification of pyrocatecase, which comprises an exhaustive dialysis, we found that our earlier experiment had been incomplete. As reported above the enzyme "pyrocatecase" is one of the specific enzymes, having ferrous ion as an important component of the enzyme like "homogentisicase" (*5*) or "aconitase" (*6*).

The enzyme which catalyzes the conversion *cis-cis* muconic acid into β-ketoadipic acid is obtained in the cell-free state, but the nature of this enzyme is not known precisely at present except the facts that the enzyme reaction can be carried out without oxygen uptake and is inhibited by the presence of excess iron sulfate and by silver nitrate (end concentration, $10^{-4}M$).

SUMMARY

1. The further study of the pyrocatecase catalyzing breakdown of catechol has shown that one of the essential compentents of this enzyme is ferrous ion.

2. The end product of pyrocatecase reaction may be *cis-cis* muconic acid as reported earlier. By use of dried cells adapted to catechol, and cell extracts, *cis-cis* muconic acid was shown to be converted to β-ketoadipicacid.

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The Journal of Biochemistry, Vol. 33, No. 3.

# METABOLISM OF TYROSINE

## III. RELATION BETWEEN HOMOGENTISICASE, FERROUS ION AND L-ASCORBIC ACID IN EXPERIMENTAL ALCAPTONURIA OF GUINEA PIG

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(Received for publication, June 2, 1951)

Previously we have established that homogentisic acid is an intermediate in the course of tyrosine metabolism in *Pseudomonas sp.* using the successive adaptation method (*1*) and also have isolated from rabbit liver and partially purified an enzyme which catalyzes the oxidative breakdown of homogentisic acid to fumaric and acetoacetic acids (*2*, *3*). We designated this enzyme as homogentisicase. Besides the ordinary coenzyme, homogentisicase requires another active principle, ferrous ion.

Sealock *et al.* (*4*) showed that the oral administration of tyrosine to scorbutic guinea pig resulted in the excretion of phenolic compounds, chiefly of homogentisic acid. However, the excretion of these metabolites was not observed if animals received L-ascorbic acid. This fact suggests that there is a connection between the function of L-ascorbic acid and normal metablism of L-tyrosine.

In the previous report (*3*) we discussed that the analysis of the interaction between L-ascorbic acid and ferrous ion in homgentisic acid metabolism would throw some light on this problem.

As reported below, we tested the homogentisicase activity in scorbutic liver and liver of guinea pig treated with $\alpha\alpha'$-dipyridyl and it was confirmed that our previous postulation was correct.

### EXPERIMENTAL

*Scorbutic Animals*—Guinea pigs* weighing approx. 300 g. were

The compendium of this article was delivered at the Annual Meeting of the Vitamin Society of Japan in April 1951 at Osaka University.

* Male guinea pigs were used in our experiment.

fed with a scorbutic diet, beancurd refuse, *ad libitum* for 20 days. The control guinea pigs were given a daily intraperitoneal supplement of 10.0 mg. of L-ascorbic acid. Both animals were received an oral administration of 500 mg. of L-tyrosine. An animal was not regarded as scorbutic until it had lost considerable weight and at autopsy showed considerable hemorrhage about the thighs.

*Animals Treated with αα'-Dipyridyl*—Guinea pigs weighing approx. 300 g. were fed with the above scorbutic diet *ad libitum*, and received daily intraperitoneal injections of 10.0 mg. of L-ascorbic acid and 15.0 mg. of αα'-dipyridyl solutions plus 500 mg. of L-tyrosine orally.

*Preparation of the Enzyme*—The animals were killed by a blow on the head. The liver was removed and homogenized with 1.5 volumes of 0.04 *M* phosphate buffer (pH 7.2) in a refrigerating high speed mixer; and centrifuged; the supernatant was heated at 55° for 5 minutes; and again centrifuged; the supernatant was used as the enzyme solution. Ferrous ion was not separated from the enzyme by these treatments.

*Manometric Measurement*—The oxygen consumption was measured in Warburg flasks at 38°. 0.2 ml. of 20 per cent KOH plus a filter paper wick was used in the center cup.

*Determination of Homogentisic Acid*—As Brigg's method is not specific, we used the manometric estimation with homogentisicase. The purification of this enzyme was described in the previous report (*3*). The procedure is as follows: urine is extracted with ether after acidifying with 10 per cent sulfuric acid, added 0.1 *M* phosphate buffer (pH 7.2) to this ether extract, and ether is evaporated under reduced pressure. An aliquot of this solution is used as substrate. Since oxygen uptake is calculated to be 2 atoms per mole of homogentisic acid (*3*), the homogentisic acid content in urine can be computed from oxygen consumption.

## RESULTS

*Scorbutic Guinea Pig*—As shown in Fig. 1, the homogentisicase activity in scorbutic guinea pig liver is remarkably lower than the control, but it is recovered on addition with ferrous sulfate or L-ascorbic acid to the enzyme solution. Ferrous ion is by far more effective than L-ascorbic acid. Since the enzyme preparation used may contain ferric ion which is reduced readily to ferrous ion on addition of L-ascorbic acid, the enzymatic activity is increased in the presence of the latter. It is considered that the action of L-ascorbic acid is ascribed to the maintenance of

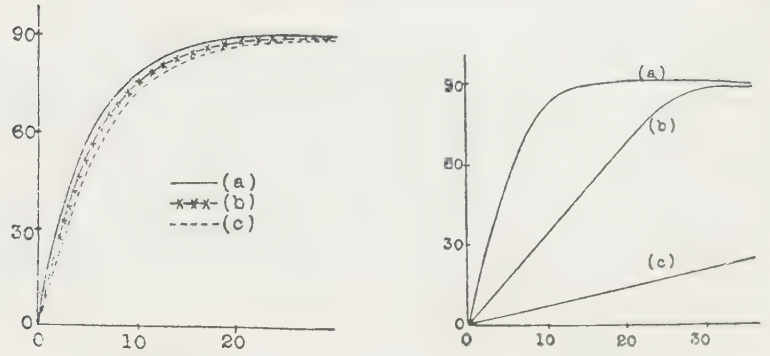


FIG. 1. The effects of L-ascorbic acid and ferrous ion on the homogentisicase activity in livers from normal and scorbutic guinea pigs.

The incubation mixture contains 1 ml. of enzyme solution and 0.2 ml. of 0.02 *M* homogentisic acid. (a) contains 0.2 ml. of $2 \times 10^{-3}$ *M* ferrous sulfate and (b) contains 0.2 ml. of $2 \times 10^{-3}$ *M* L-ascorbic acid. To all vessels 0.04 *M* phosphate buffer (pH 7.2) is added so as to establish a final volume of 2.2 ml. pH of each vessel is adjusted to 7.2. Gas phase: air.

L-Ascorbic acid or ferrous sulfate is tipped in simultaneously with the substrate.

Curve (a); with ferrous sulfate.
Curve (b); with L-ascorbic acid.
Curve (c); without additions.

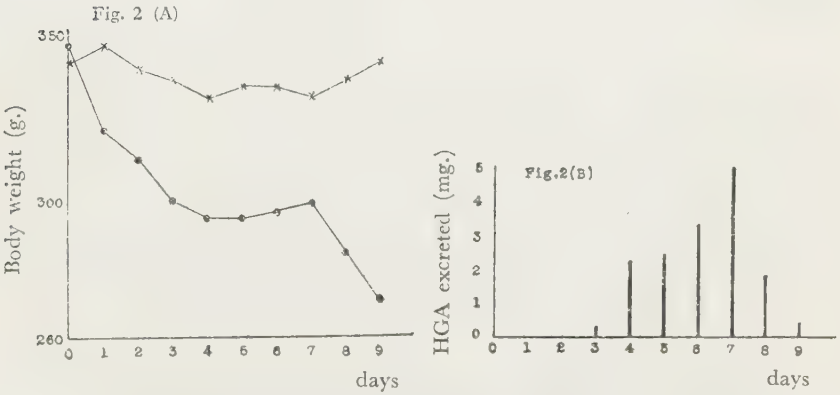


FIG. 2 (A). The loss of body weight in $\alpha\alpha'$-dipyridyl-treated guinea pig.
x——x normal guinea pig.
o——o $\alpha\alpha'$-dipyridyl-treated guinea pig.

FIG. 2 (B). The urinary excretion of homogentisic acid (HGA) in $\alpha\alpha'$-dipyridyl-treated guinea pig.

ferrous ion level in animal body owing to its reducing action.

*αα′-Dipyridyl-Treated Guinea Pig*—It is seen from Fig. 2 that despite of the supplement of L-ascorbic acid, *αα′*-dipyridyl-treated guinea pigs lost considerable weight and excreted phenolic compounds in urine following the administration of L-tyrosine.

It was confirmed by paper chromatography that the most part of these phenolic compounds excreted in the urine was homogentisic acid. The developing agent used was a trinary mixtures of butanol, isopropanol, and water (4:1:1). Homogentisic acid was detected according to Brigg's method.

As shown in Fig. 2 (B), in *αα′*-dipyridyl-treated animals, the excretion of homogentisic acid begins on about the 4th day after the admisteration of L-tyrosine. The amount of homogentisic acid excreted in urine is increased gradually and reaches its maximum on the 6–7th day. After this period, the amount of homogentisic acid excreted continues to decline. Finally this acid ceases to be excreted in urine, but urin colors red-brown. Little, if any, homogentisic acid is excreted in the control animals.

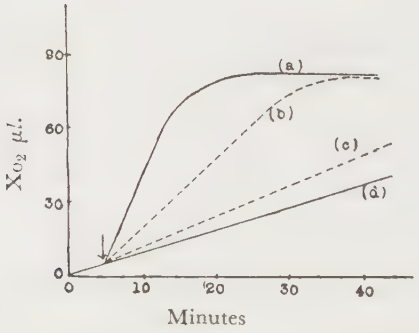


FIG. 3. The effects of L-ascorbic acid and ferrous ion on the homogentisicase activity in the liver from *αα′*-dipyridyl-treated guinea pig.

Curve (a); addition of 0.2 ml. of $2\times10^{-3}$ *M* ferrous sulfate

Curve (b); addition of 0.2 ml. of $2\times10^{-3}$ *M* ferric sulfate and 0.2 ml. of $2\times10^{-3}$ *M* L-ascorbic acid.

Curve (c); addition of 0.2 ml. of $2\times10^{-3}$ *M* L-ascorbic acid.

Curve (d); control (no addition).

Substrate: 0.2 ml. of $2\times10^{-2}$*M* homogentisic acid. Final volume of each vessel was 2.2 ml. L-Ascorbic acid or ferrous sulfate was tipped in 5 minutes after the addition of the substrate.

The homogentisicase activity of the liver of experimental groups is also remarkably lower than the control (Fig. 3). Addition of ferrous sulfate to enzyme solution prepared causes a marked increase of its activity, but L-ascorbic acid cannot recover its activity.

These results indicate that L-ascorbic acid does not act directly, but indirectly in the breakdown of homogentisic acid. And it is supposed that the low activity of homogentisicase in $\alpha\alpha'$-dipyridyl-treated animals is due to the general iron deficiency and hence it is not recovered by the addition of L-ascorbic acid.

## DISCUSSION

Based on the observations previously reported (*3*), we supposed that L-ascorbic acid plays the role of a reducing agent for ferric ion to ferrous ion which is indispensable for the enzymatic breakdown of homogentisic acid in animal body. This supposition was confirmed in the above experiments.

The facts that in the liver of scorbutic guinea pig ferrous ion activates directly homogentisicase and is more effective than L-ascorbic acid, indicate that L-ascorbic acid plays a role of maintaining the level of ferrous ion in liver. Furthermore, the fact that the low activity of homogentisicase in $\alpha\alpha'$-dipyridyl-treated guinea pig liver is recovered only by the addition of ferrous ion and not by the addition of L-ascorbic acid, shows that the treatment with $\alpha\alpha'$-dipyridyl caused a decrease of iron level in the animal body.

In $\alpha\alpha'$-dipyridyl-treated animals, the more iron is reduced, the more is this excreted in urine combined with $\alpha\alpha'$-dipyridyl. In the course of this treatment the urine of these animals colored red-brown, the color being especially remarkable in the last stage. This color can be taken to account for the excretion of ferrous ion-$\alpha\alpha'$-dipyridyl complex in urine. It is, therefore, considered that the administration of $\alpha\alpha'$-dipyridyl had caused a decrease of the general iron level in liver.

## SUMMARY

1. The activity of homogentisicase was found to be remarkably lower in the liver of scorbutic guinea pig than in the liver of control animals. This low activity could be recovered by the addition of ferrous ion or L-ascorbic acid, the addition of the latter having proved to be far less effective.

2. In the guinea pig treated with $\alpha\alpha'$-dipyridyl, homogentisic acid is excreted in urine following the administration of L-tyrosine.

3. The homogentisicase activity in liver of guinea pigs treated with $\alpha\alpha'$-dipyridyl, is remarkably lower than the control. This low activity is recovered only when ferrous ion in added to the enzyme solution. L-ascorbic acid has no effect.

4. A function of L-ascorbic acid is animal body is discussed.

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# HISTAMINE CONTRACTION OF A GUINEA PIG SMOOT MUSCLE AT LOWER TEMPERATURES WITH REFERENCE TO ADSORPTION ENERGY

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(Received for publication, June 22, 1951)

Despite the extensive studies on histamine, especially on its mode of action in connection with recent synthetic antihistaminic compounds, there still remains much to be solved. As to the mechanism of histamine antagonism, Wells, Morris, Bull and Dragstedt (1945) (*1*) assumed, from their blood pressure observations in dogs, that benadryl is adsorbed on the sites of action of histamine, where the dose-effect relationship for benadryl can be expressed by the Langmuir's adsorption equation. Little is known, however, regarding the adsorption of histamine even to simple proteins (*2*)*, although there are extensive works on the adsorption to proteins of vairous other compounds (*3*). The present study concerns the histamine contractions of the guinea pig smooth muscle at temperatures below 37°, with an attempt to calculate the adosrption energy of histamine to the muscle**. There may be some arguments against the idea of considering the histamine contraction as the result of simple adsorption of this substance on the surface of the muscle, as some chemical reactions might be involved. However, the relaxation of contraction by washing clearly indicates the reversibility of this action. The term "adsorption" may be reasonably applied to the reversible phenomenon such as the histamine contraction in question.

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* By the method of Klotz (*J. Am. Chem. Soc.* **68** 1486 (1946)) S. Nagakura in our chemical laboratory recently found no adsorption of histamine to horse serum albumin.

** T. Shimanouchi, Chemical Laboratory, Faculty of Science, is responsible for the theoretical treatment of the adsorption energy presented in this paper.

## EXPERIMENTALS

The usual Magnus technic was used, consideration being taken to recent quantitiative aspects of Chen, *et al.*(*4*) and Rocha E Silva and Beraldo (*5*). A small bath of 100 ml. capacity with constant flow of oxygen bubbles was kept in a large thermostat, a thermometer being fixed close to the muscle strip for exact measurment of water temperature surrounding the muscle. Strip of about 5 cm. in length of the ileum from the guinea pig* weighing about 300–350 g. was suspended in the Tyrode solution prepared according to Landau and Gay (*6*). The strips gave a minimum response to 0.002–0.003 $\mu$g./ml. histamine dihydrochloride, whereas a maximum contraction to about 0.1–0.5 $\mu$g./ml. was observed. Immediately after the stable contraction with no spontaneous relaxation was reached in a test, the strip was completely washed with a sufficient amount of the Tyrode solution. The next contraction of the same sample was observed after 5–10 minutes, rest in the newly replaced Tyrode solution. Temperatures at which the observations were made were; 37°, 33°, 29°, and 25°, respectively.

## RESULTS AND DISCUSSIONS

Some typical results at temperatures 33° and 29° which were well reproducible, are given in Fig. 1 and Table I.

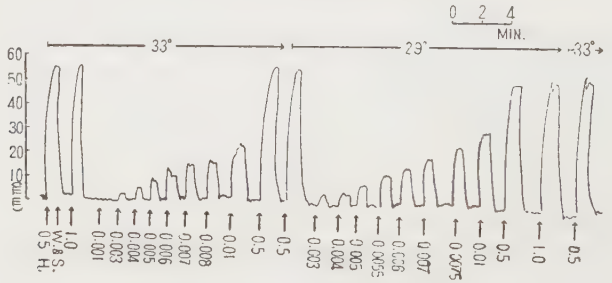


FIG. 1. Contractions of an intestinal strip of a guinea pig with various histemine concentrations in a bath at 33° and 29°. (See Table I)

H: histamine dihydrochloride, $\mu$g./ml.

W & S: washed and rested for 5–10 min.

* It may be added here that no regular results were obtained with a rabbit intestine which is much less histamine-sensitive.

TABLE I

*The Relative Contractions of the Smooth Muscle Strip of the Ileum of the Guinea Pigs in Various Histamine Concentrations at 33° and 29°*

| Temperature of bath | Concn. (C)† | Relative length ($l$)†† | $\theta$††† |
|---|---|---|---|
| 33° | 0.5 | 55 | |
| | 1.0 | 55 | |
| | 0.001 | 0 | |
| | 0.003 | 3 | 0.055 |
| | 0.004 | 5 | 0.091 |
| | 0.005 | 8 | 0.145 |
| | 0.006 | 10 | 0.181 |
| | 0.007 | 13 | 0.236 |
| | 0.008 | 15 | 0.272 |
| | 0.01 | 22 | 0.4 |
| | 0.5 | 55 | |
| 29° | 0.5 | 55 | |
| | 0.003 | 4.5 | 0.082 |
| | 0.004 | 5 | 0.091 |
| | 0.005 | 9 | 0.163 |
| | 0.0055 | 12 | 0.218 |
| | 0.006 | 15 | 0.273 |
| | 0.007 | 19 | 0.345 |
| | 0.0075 | 24 | 0.435 |
| | 0.01 | 31 | 0.563 |
| | 0.5 | 52 | |
| | 1.0 | 54 | |
| 33° | 0.5 | 55 | |

† $\mu$g./ml. histamine dihydrochloride in the bath.

†† Relative height (contraction) of the strip recorded on a kymograph at a given histamine concentration.

††† $\theta = \frac{l}{l_0}$ ($l_0$, maximum contraction. In this case $l_0 = 55$)

The experiment performed at lower temperatures resulted increasod magnitude of contraction, indicated as the length* of contracted strip ($l$) at a certain histamine concentration.

This temperature effect, which was also the case for all the other results obtained in the present experiments, may be caused by various

* Relative length as measured on the kymogram.

factors. One of the main factors would be the effect of temperature on histamine adsorption. The experimental results can be explained satisfactorily if we assume that the grade of contraction, as shown by the grade of shortening of the strip, be proportional to the number of sites of action on smooth muscle where histamine molecules become adsorbed. If $\theta$ denotes the ratio of the relative length of the contracted strip $l$ at a given concentration C to the maximum contraction $l_0$, shown as the maximum height on the kymogram, this will represent the ratio between the amount of histamine adsorbed at the concentration C and the maximum amount of histamine adsorbed, and would be expressed by the following adsorption isotherm (*7*, *8*):

$$\frac{\theta}{1-\theta}=\frac{C}{C_0} \qquad \text{(Eq. 1)}$$

where $C_0$ is a constant in relation to the energy of adsorption E per mole:

$$C_0=A\exp\left(-\frac{E}{RT}\right) \qquad \text{(Eq. 2)}$$

Where R is the gas constant, T the absolute temperature and A, a constant.

Fig. 2 shows a fairly close conincidence of the values given by Eq. 1 with the experimental figures. The linear relationship, well obtained at lower concentrations, may be interpreted as indicating that the adsorption of histamine molecules on the receptive sites of the muscle is responsible for the histamine contraction. Obviously, the points in Fig. 2 are not strictly on straight lines, but the deviation at the lowest concentration may be considered as being within the limit of experimental error. The considerable deviation at higher concentrations, however, may presumably be due to heterogeneity of the sites of histamine adsorption. A far more complex theoretical treatment, which would cover the heterogeneity of active sites, will give the same conclusion as to the adsorption energy. This was the reason why the linear part was exclusively treated.

By comparing the slopes of the two straight lines in Fig. 2 the energy of adsorption can be calculated using Eq. 2*. A series of temperature dependency experiments gave the values tabulated in Table II.

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* If we denote $C_0$ measured at temperatures $T_1$ and $T_2$ by $C_{01}$ and $C_{02}$ respectively, the adsorption energy can be calculated from the following equation:

$$\frac{C_{01}}{C_{02}}=\exp\left[\frac{E}{R}\left(\frac{1}{T_2}-\frac{1}{T_2}\right)\right]$$

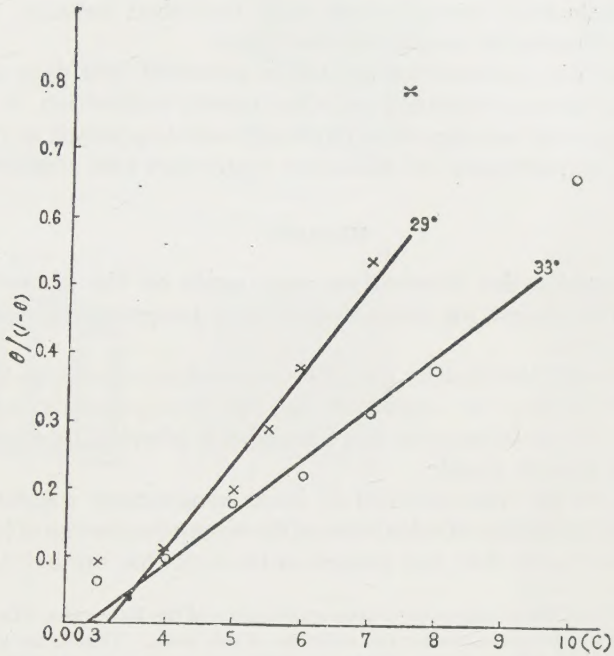


FIG. 2. A linear relationship between histamine concentration (c) ($\mu$g./ml.) and $\theta/(1-\theta)$ at 33° and 29°.

TABLE II

*Temperature Range and Adsorption Energy* (E) *of Histamine (Calculated)*

| Experiment | Temperature range | E (Kcal/Mol.) |
|---|---|---|
| 1 | 33°–29° | 12 |
| 1a | 29°–25° | 11 |
| 2 | 37°–33° | 8 |
| 2a | 33°–29° | 6 |
| 3 | 33°–29° | 14 |
| 4 | 37°–33° | 11 |
| 5 | 37°–33° | 8 |
| 6 | 37°–33° | 10 |
| 7 | 33°–29° | 7 |

Note: Experiments 1, 1a and 2, 2a were carried out with the same strips, respectively.

The calculated energy varies with individual animals, but well within the reasonable magnitude and range.

Although the mathematical treatment presented here does not cover all the mechanism concerned with the muscle contraction, it may be worth while as an attempt on a physicochemical approach to the elucidation of the mechanism of histamine contraction and antagonism.

SUMMARY

The quantitative observations were made on the histamine contraction of the guinea pig ileum strip at lower temperatures with various concentrations.

The results obtained by the Magnus technic at various histamine concentrations can be explained by the Langmuir's adsorption equation with the assumption that histamine is adsorbed on to the active sites of the smooth muscle.

Based on the data obtained at lower temcratures suggestion was made on the possibility of calculation of the adsorption energy of histamine to the muscle strip that was gauged to be somewhat around 10 K cal.

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